1985

Production and bioassay of an endogenous myocardial depressant

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PRODUCTION AND BIOASSAY OF AN ENDOGENOUS MYOCARDIAL DEPRESSANT

Iowa State University PH.D. 1985

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Production and bioassay of an endogenous myocardial depressant

by

Wayne Hillery Terry

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Physiology and Pharmacology
Major: Physiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1985

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LIST OF ABBREVIATIONS

Symbol	Definition
a	artery or arterial
A-V	arterial-venous difference
AT	active or developed isometric tension
BW	body weight
BSA	body surface area
BSA	bovine serum albumin
C	temperature expressed in Celsius units
cm	centimeter
Contr.	contraction
CVP	central venous pressure
DUR	duration
dL/dt	change in length per change in time
+dL/dt	maximum rate of muscle shortening; contraction velocity
-dL/dt	maximum rate of muscle lengthening; relaxation velocity
dT/dt	change in tension per change in time
+dT/dt	maximum rate of tension developed
-dT/dt	maximum rate of tension decline or relaxation of tension
ECG	electrocardiogram
F	frequency of electrical stimulation
F12	stimulus frequency of 12/minute
F60	stimulus frequency of 60/minute
g	grams

G	gravity
g/mm^2	grams per square millimeter
g/ml	grams per milliliter
$g/sec/mm^2$	grams per second per square milliliter; units expressing the rate of tension developed per muscle cross-sectional area
I.D.	inside diameter
IV	intravenous
kg	kilogram
K-H	modified Krebs-Henseleit solution
L	latency
LAT	latency
Lmax	muscle length at which the maximum tension is formed
M	molar solution
MDF	myocardial depressant factor
MABP	mean arterial blood pressure
Max.	maximum
mg	milligram
mm Hg	millimeters of mercury; units for expressing pressure
ml	milliliter
mOsm/kg	milliosmols per kilogram
meq/l	milliequivlents per liter
$mm/sec/mm^2$	millimeters per second per square millimeter; units used to express velocity or change in muscle length per muscle cross-sectional area
msec	millisecond

ml/hr	milliliters per hour
μμg	micromicrogram
MW	molecular weight
N	normal solution
nM	nanomole
P	pressure; muscle load or weights added to the isotonic lever system which the muscle must overcome prior to shortening
p<	statistical probability less than (some number)
Po	maximum load or the weight added to the isotonic lever system which just prevents muscle shortening
P/Po	normalized afterload or the muscle load divided by the maximum load
pH	measure of solution acidity or alkalinity
PaO_2	arterial oxygen tension
$PaCO_2$	arterial carbon dioxide tension
PO_2	partial pressure of oxygen
psi	pounds per square inch
RT	resting tension
Relax.	relaxation
S.D.	standard deviation
SMA	superior mesenteric artery
S-T	interval in the electrocardiogram from the S wave to the T wave
TEMP	temperature
TMVC	time from stimulus to maximum velocity of contraction

	(+dL/dt)
TMVR	time from stimulus to maximum velocity of relaxation (-dL/dt)
TPC	time from stimulus to peak of the contraction or shortening
TPT	time from stimulus to peak developed tension
TRLX	time from peak of contraction or peak tension to completion of contraction
U	units
v	venous
Vc	maximum velocity of contraction; +dL/dt
Vr	maximum velocity of relaxation; -dL/dt
XSA	cross-sectional area

INTRODUCTION

Clinical shock is a complex pathophysiological process. In 1892, Samuel Gross defined shock as "a rude unhinging of the machinery of life". Although his definition is still valid, a more current definition would define shock as an altered physiological state characterized by a decreased effective blood volume. This loss of effective blood volume may be caused by various mechanisms. Examples of these mechanisms include blood loss, loss of smooth muscle tone, or a hypoeffective heart. The body's ability to maintain blood pressure and adequate cardiac output is impaired which compromises adequate perfusion of tissues and ultimately leads to cell damage and death.

Shock has been classified into categories based on the primary cause of the altered state. These common categories include endotoxic, hemorrhagic, neurogenic, and burn shock. Each form of shock may be initiated by altered function in one body area or organ but all may progress to a systemic derangement of multiple functions and failure to maintain tissue perfusion. When sufficient function has been altered, the body can no longer compensate and a state of irreversible shock and eventual death occurs. Physiologists and clinicians continue to debate the role of various mechanisms in this irreversible state and especially the role of peripheral versus central collapse of the cardiovascular system. Is the depressed myocardial function observed in shock secondary to deranged peripheral vascular or microvascular collapse or does impaired myocardial function fail to sustain adequate peripheral perfusion pressure? Both processes can initiate positive feedback

circuits and ultimately produce a state of irreversible shock.

Cardiac failure in terminal shock is widely accepted, but the role of central failure earlier in the process is controversial. Excellent reviews of the role of the myocardium in shock have been written by Lefer (1974,1979), Hess et al. (1983), and Okada et al. (1985). Wiggers and Werle (1942) first reported and Crowell and Guyton (1961, 1962) later confirmed that cardiac performance decreases with time during severe hypotension. Crowell and Guyton established the shifts in ventricular function curves during hemorrhagic shock which have been interpreted as a progressive impairment of myocardial contractility. As the duration of shock increased, atrial pressure increased while cardiac output progressively declined.

Functional and morphological changes in the myocardial cell in shock are well-documented. An in-depth review of the morphological changes is not appropriate to this paper. However, Martin and Hackel (1966) and Hackel et al. (1974) have reported typical changes which include mitochondrial displacement, hypercontraction of the sarcomeres, fragmentation of the Z-bands, and abnormal organization of the myofilaments.

Hemorrhagic shock has been reported by Forrester et al. (1972) to decrease pump performance but not necessarily alter the maximum velocity of contraction which is a measure of contractility. Significant reduction of both stroke volume and cardiac output was reported by Siegel and Downing (1970) and Forrester et al. (1972) but no long-term effects on either heart rate or left ventricular end diastolic pressure were observed. Goldfarb (1982) and Downing (1983) have reviewed many of

the functional changes in shock. The subcellular function of cardiac tissue has also been reviewed by Hess and Krause (1979).

Cardiac failure in shock has been attributed to many different factors. The list of these factors includes acidosis, decreased coronary blood flow, the effects of clotting factors which cause 'sludging of blood', hypoxia, altered hormone function, and the production of endogenous factors which depress myocardial function.

The role of these endogenous factors has been very controversial. Several excellent reviews of shock factors have been published by Lefer (1973, 1978, 1979), Goldfarb (1979), and Haglund (1983). Haglund has defined a shock toxin as a substance that is released into the general circulation in shock and which then exerts a deleterious effect on the body. These substances are produced in specific regions or tissues in the body and enter the systemic circulation via the lymphatics or directly into the blood. They are transported to and interact at the target organ(s) where they alter function. Examples of lost function include loss of smooth muscle tone and impaired myocardial contractility which lead to peripheral vasculature collapse and decreased cardiac output, respectively.

Haglund has proposed four criteria necessary for defining a shock toxin. First, there should be no production of the substance in a healthy individual. It must be possible to isolate and identify the unique biochemical composition. Isolated material from a shock individual must produce toxic effects in a healthy individual. Finally, a shock toxin should be common to multiple categories of shock.

Myocardial depressant factor (MDF) is the best known and most

extensively studied of these shock factors. MDF was first described by Brand and Lefer (1966). It has been found in all types of shock in the plasma of virtually all species which have been evaluated, as reported by Lefer (1974). Lefer and Spath (1974) have shown that MDF is produced in shock as blood flow to the pancreas is compromised. Ferguson et al. (1972) have shown that all of the necessary constituents for production are present in the pancreas. The MDF is released by the pancreatic cells when sufficient insult of shock is present. The MDF is transported either via the lymphatic channels or enters the circulation directly.

There have been three identified effects of MDF on systemic function. First, Lefer (1970) reported a negative inotropic effect which was measured as depressed peak developed tension by feline papillary muscles. Glucksman and Lefer (1971) subsequently observed a pronounced vasoconstrictor effect of strips of the superior mesenteric artery but not other central and peripheral vessels. Finally, Lefer and Blattberg (1968) reported that MDF inhibited the phagocytic activity of fixed macrophages in the liver. The three effects all contribute to a positive feedback phenomenon which may ultimately contribute to irreversible shock. As cardiac output falls, the pancreas becomes hypoperfused and MDF is formed. It is released into the circulation where it further depresses cardiac output and causes a potent constriction of the vasculature which nourishes the pancreas. This further compromises pancreatic blood flow. Inhibition of the macrophagic activity may prevent clearance or reduction in the levels of MDF.

MDF has not been completely purified but has been separated by gel chromatography and is believed to have a molecular weight between 500-1000, as reported by Lefer and Martin (1970). Green et al. (1977) have determined that the material is a peptide composed of glycine, serine, glutamic acid, and an unidentified fourth molecule. Although Lefer (1970) reported only nanogram levels in the plasma during shock, the negative inotropic effects have been consistently observed and are potent. The inotropic effect of MDF has been reported by Lefer and Rovetto (1970) to be independent of an effect on either resting membrane potential or the amplitude of the action potential. They did observe a longer duration of the action potential and reported that excess calcium can antagonize the effects of MDF.

There has been substantial confirmation of MDF and reports of MDF-like activity by others. Rogel and Hilewitz (1978) have correlated *in vivo* and *in vitro* changes believed to be caused by MDF. Fisher et al. (1973) established a dose response curve for MDF which was approximately linear. Manning et al. (1980) measured MDF levels in ante and postmortem blood of trauma and disease victims. Levels were increased in those patients with compromised pancreatic blood flow or pancreatitis. The plasma concentration increased in the terminal stages of life. Lee et al. (1981) observed depressed myocardial function in patients with acute pancreatitis and postulated that this response was due to MDF. Their study emphasizes the potential role of a depressant factor in disease processes other than shock.

The evidence for a role of MDF is very strong. However, attempts to confirm the presence of MDF or central cardiac depression in other

laboratories have not always been successful. Hinshaw (1979) has reviewed the extensive studies undertaken in his laboratory to detect depressed myocardial function in response to endotoxin shock or occlusion of the superior mesenteric artery (SMA). Despite total occlusion of the SMA or 20 hours of endotoxin shock, no depression could be detected. His methods often employed cross-perfusion of a working but isolated heart where temperatures less than 37 C might be expected. Wilson et al. (1973) assessed dialysates of serum obtained from hypotensive dogs with rabbit papillary muscles. No depression in the peak tension or rate of development was observed. Neither their methods for dialysis nor for the bioassay system were described. Differences in methods could have accounted for their observation. Chimoskey and Bohr (1965) compared the myocardial performance of papillary muscles from rats subjected to hemorrhagic shock and from control rats. No impaired contractility was observed. However, their muscles were not tested in the shock plasma and MDF effects are known to be at least partly reversible. Adams et al. (1981) evaluated atrial muscle from burned guinea pigs for changes in contractility. Again, no change in isometric tension was produced but the rate of relaxation was significantly impaired. The experimentation was conducted at 30 C. Urschel et al. (1972) evaluated both fresh shock plasma and the effects of endotoxin on feline papillary function at conditions of 30 C and a frequency of 12/minute. No depression was observed.

Repeated efforts were made in our laboratory over a two year period to identify and evaluate MDF. Occasionally, a depressant was observed. After reviewing the literature it was noted that negative findings were

associated with methods that differed markedly from the protocols of Lefer. Our bioassay preparation was unique in several respects. Young canine papillary muscles were used with an isotonic preparation at 27 C and a stimulus frequency of 12/minute. Lefer had utilized feline muscles contracting isometrically at 37 C and a frequency of 60/minute. It was felt that species, age, and type of contraction were the least likely factors to obscure a depressant action. If conditions of temperature or frequency could alter muscle responsiveness to the depressant, then that observation might help to reconcile the controversy concerning MDF.

The following studies were conducted. First, a study was conducted to confirm the presence of an endogenous depressant in the plasma of canines in hemorrhagic shock. Although prior studies have evaluated MDF effects in the intact dog, none had confirmed the effects on isolated canine papillary muscle. Second, the production and presence of a depressant in homogenates of pancreas were evaluated. The final studies were conducted to assess the effects of temperature and frequency on cardiac muscle function and on the response of the muscle to the endogenous depressant.

PRODUCTION AND BIOASSAY OF A MYOCARDIAL DEPRESSANT IN SHOCK PLASMA

Introduction

The role of a myocardial depressant substance in shock remains controversial. Considerable information has been published on the various parameters, such as cardiac output, blood pressures, oxygen consumption, etc., which are altered in a shock state. However, extensive physiological information was not reported in the previous studies on MDF by Lefer (1973, 1974, 1979) and Goldfarb et al. (1979). The evidence accumulated by Lefer and others for MDF is strong and extensive, yet in independent studies by Coleman et al. (1975), Hinshaw (1979), Urschel et al. (1972) and Wilson et al. (1973), myocardial depression or indications of central cardiac failure have not been detected until very late in shock. These different conclusions reported by the investigators may have been due to variation in the procedure to generate the depressant. Therefore, a well-defined model in which dogs were subjected to hemorrhagic shock, was initially chosen to evaluate the role of an endogenous depressant substance in shock. The specific protocol for the production of the depressant in these experiments was similar to that previously reported by Lefer and Martin (1970) and based on the model of Wiggers (1950). The techniques employed in assaying depressant activity are either widely accepted in the literature or have been previously reported for in vitro assessment of myocardial function by Hembrough et al. (1978) and Hembrough et al. (1980). However, the canine derived depressant was bioassayed on isolated canine myocardium contracting isotonically. The methods employed also enabled examination of muscle function over a range of afterloads and assessment of

of relaxation velocities and intervals of time in the contractile cycle in addition to maximum load. Only limited studies by Hembrough et al. (1980) and Glassman (1980) have been reported in which whole plasma containing a depressant substance was bioassayed in vitro without antifoaming substances and with adequate oxygenation.

A series of experiments were conducted to determine whether an endogenous myocardial depressant substance could be detected in the plasma of shock animals. The specific objectives were as follows. First, dogs were subjected to hemorrhagic shock conditions which, according to reports in the literature, should have been adequate to cause the formation of a depressant. Second, various physiological parameters were recorded during the hemorrhagic shock experiments to define the shock condition. Finally, the presence of a depressant in shock plasma was determined by immediately comparing shock plasma with control plasma and a modified Krebs-Henseleit solution in a bioassay system utilizing isolated canine papillary muscle.

Methods: Hemorrhagic Shock Experiments

Methods of hemorrhage

Seven adult dogs weighing 20.5 ± 4.1 (S.D.) kg were subjected to a hemorrhagic shock procedure based on the model of Wiggers (1950). Food was withheld from each dog at least fifteen hours prior to the experiment. A light plane of anesthesia was induced with intravenous sodium pentobarbital at an initial dose not exceeding 25 mg/kg. An endotracheal tube was immediately inserted and an electrocardiogram (Lead II) recorded. Arterial blood pressure and arterial blood samples were obtained via a polyethylene cannula (Clay Adams PE 205) which was

connected to a Statham P23 transducer and then inserted into the femoral artery and advanced near the heart. A second polyethylene cannula was inserted into the right jugular vein and advanced either into the right atrium or in close proximity to the atrium, as determined at postmortem examination. Central venous pressure was then monitored with a water manometer. The left carotid artery was cannulated with a large bore (2.5 mm i.d.) cannula for infusion and withdrawal of blood (hemorrhage). This cannula was connected to a closed two liter siliconized Plexiglas reservoir to which 1000 units of heparin had been added. Flow into and out of the reservoir was regulated by varying the pressure in the reservoir with a hand pump and valve. The reservoir was maintained at 37 C by a water bath. Calibration marks on the reservoir were used for periodically determining the shed blood volume. Recordings of the arterial blood pressure, mean arterial blood pressure, respiratory rate, and electrocardiogram were made on a Beckman Model R611 polygraph. Rectal temperature was continuously monitored with a YSI Model 47 thermometer. Body temperature was maintained with a heating plate placed beneath the dog. Heparin sodium (500 U/kg) was administered intravenously immediately after all preparatory work was completed and at least 20-30 minutes prior to hemorrhage. A schematic of the preparation is shown in Figure 1.

Some of the dogs were maintained on a Bird Mark VII respirator used in an assist mode either periodically during the procedure or throughout the experiment. All dogs were ventilated with either compressed air or room air. Oxygen consumption was determined in the remaining dogs concurrently assisted by the respirator. However, the method employed

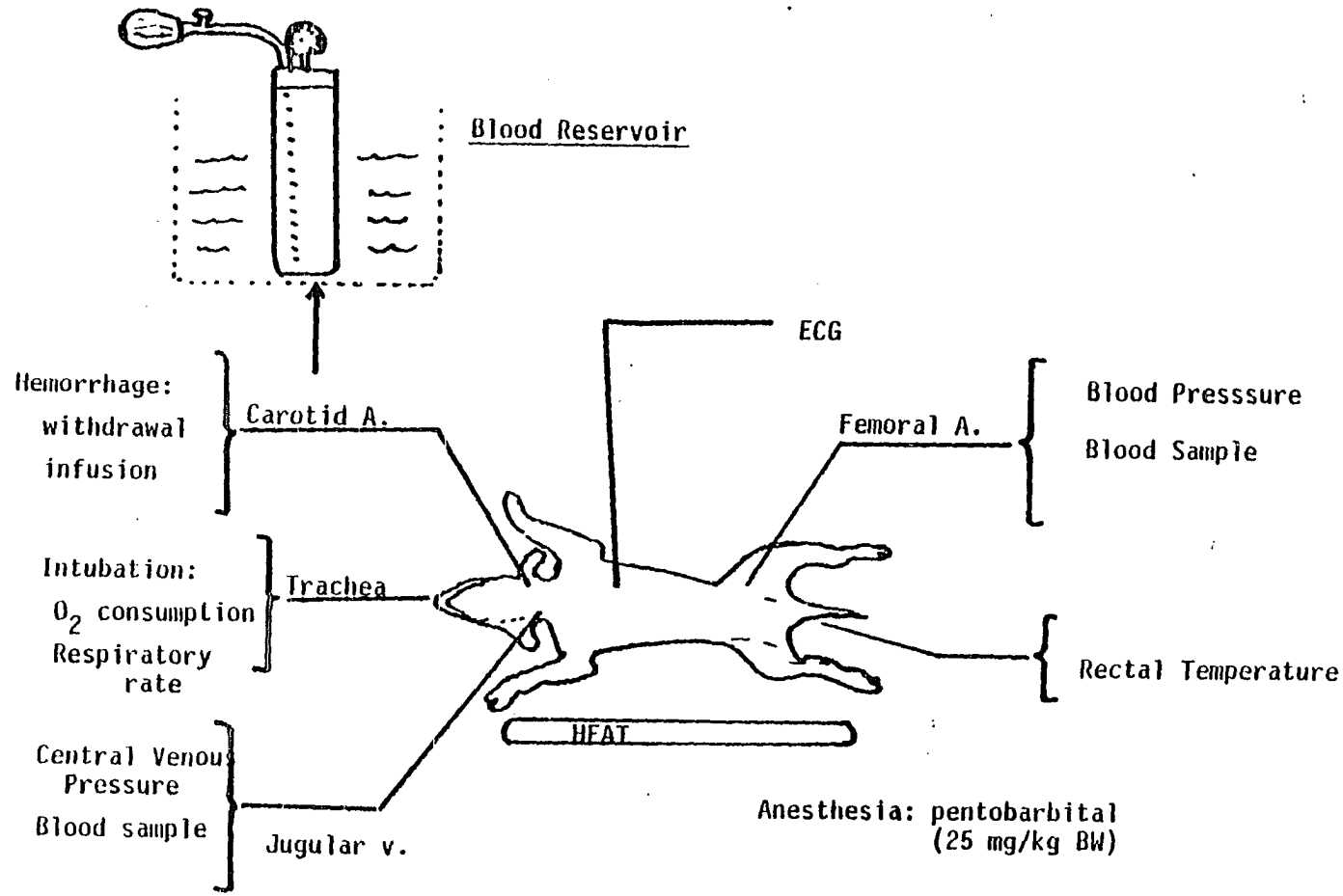


Figure 1. Schematic of the experimental preparation: hemorrhagic shock experiment

for simultaneously measuring oxygen consumption during assisted ventilation was later determined to be invalid.

Hemorrhage was induced by opening the carotid cannula and allowing the blood pressure to equilibrate with the reservoir pressure which was maintained at 40 mm Hg. Shed blood volume was determined from the calibration marks on the reservoir and recorded. The dog was maintained in the hypotensive state until 40% of the maximum shed blood volume had been taken back up by the animal. All remaining shed blood was then reinfused over a 15-20 minute period by increasing the pressure in the reservoir. The dog was then monitored until such time as his mean arterial blood pressure declined to less than 60 mm Hg. A pressure decline to less than 60 mm Hg. MABP is generally considered to be indicative of irreversible shock. Exsanguination was rapidly accomplished by bleeding from the carotid cannula into the reservoir and via removal by syringe from the femoral artery.

A gross postmortem examination was then performed to identify any overt pathological conditions, the placement of the cannulae, and the general appearance of the major organs of the chest and abdomen.

The initial 200-300 ml of shed blood withdrawn during the hypotensive period were removed from the reservoir system, centrifuged at 2000 X G for 20 minutes at 4 C, and the plasma removed. This plasma was either used for bioassay (Control plasma) on a papillary muscle or processed as described later. The cellular fraction was then resuspended in a volume of saline equivalent to the plasma removed and returned to the reservoir. Plasma obtained at the final exsanguination (Shock plasma) was immediately iced and also prepared for bioassay or

further processing.

Physiological measurements were made and samples obtained during five sample periods during the shock procedure. These periods are defined as follows:

Period I = prior to hemorrhage but at least 20 minutes after all preparatory work was completed;

Period II = 30 minutes after reaching a stable mean arterial blood pressure (MABP) of 40 mm Hg following the initial hemorrhage;

Period III = not more than 30 minutes prior to reinfusion of all remaining shed blood;

Period IV = 30 minutes after reinfusion of all shed blood

Period V = just prior to exsanguination when the MABP was 60 mm Hg or at a higher pressure where the decline in MABP was so rapid as to prohibit sampling without risk of the dog prematurely dying.

During each sample period, rapid tracings (25 mm/sec) were made on the polygraph, blood samples were taken from the arterial and venous cannulae, central venous pressure was recorded, and oxygen consumption was measured.

Parameters monitored were arterial blood pressure; central venous pressure; electrocardiogram Lead II (ECG); respiratory rate; rectal temperature; oxygen consumption (limited); red and white blood counts;

plasma protein concentration and osmolarity; hemoglobin; packed cell volume; and plasma sodium, potassium, and calcium concentrations. Arterial and venous samples were also analyzed for pH, pCO_2, pO_2, HCO_3^-, total CO_2, and base excess/deficit.

Analysis of Samples

Arterial and venous blood samples were collected in heparinized syringes or Vacutainers during each sampling period and assayed as follows. Plasma protein was determined with an American Optical hand refractometer. Hemoglobin was measured by the cyanomethemoglobin method on a Beckman Spectronic 20 spectrophotometer. A Coulter Counter Model M430 was used to determine the red and white blood cell counts. Anaerobic blood samples were immediately iced and the blood gas parameters measured with an International Laboratories Model 513 Blood Gas Analyzer within fifteen minutes of withdrawal. Osmolarity of all samples was measured at the conclusion of the experiment with an Advanced Osmometer. Plasma samples were also analyzed for sodium, potassium, and calcium by several methods. Samples were refrigerated overnight and forwarded to the Veterinary Medicine Clinical Pathology Laboratory for analysis with an International Laboratories Model 343 flame photometer the next day. Aliquots of plasma were also frozen and later assayed for sodium and potassium with a Technicon Autoanalyzer, as per Technicon Method File N-20B. Calcium concentration was also determined the same day of the experiment by the method described by Annino and Giese (1976).

Processing of plasma

Both control and shock plasma were processed alike. Measured volumes of plasma were iced immediately upon withdrawal from the animals. All samples were subsequently kept at 4 C except during transfer and bioassay. Those samples for bioassay were analyzed for osmolarity and calcium concentration and adjusted with 0.1 N HCl or 0.1 N NaOH to a pH of 7.4 before bioassay. All remaining volumes of each plasma were measured and the samples lyophilized for approximately 15 hours (Labconco Freeze Dryer 5). The ratio of dried weight of the sample per original volume of plasma was used as the reconstitution factor. Therefore, in all subsequent experiments, material in equivalent volumes of original material could be compared. Dried material was placed in a desiccator, containing desiccant, which was then sealed under a vacuum and stored at -10 C until used. Appropriate samples could be removed and the remaining material returned to the freezer for storage.

Results of the Shock Experiments

The shock experiments were performed in order to obtain control and shock plasma for subsequent *in vitro* bioassay and to document the physiological status of the experimental animals during the procedures. All animals utilized in this study appeared healthy prior to experimentation. Further, no conditions were found at post mortem examination which would subject the results to question. Data, unless otherwise stated, have been expressed as mean $\pm$ standard deviation of the mean (mean $\pm$ S.D.).

Characteristics of the dogs and the respective sample periods have

Table 1. Summary of data on dogs and sample periods

Seven dogs	
Body weight: 20.1 ± 4.2 kg	
Calculated blood volume: 1,704 ± 325 ml	
Percent of blood volume shed: 65.5 ± 11.3%	
Time to respective sample period (minutes):	
Period I	0 (minutes)
Hemorrhage	9.3 ± 5.3
Period II	52.7 ± 10.6
Period III	222.7 ± 68.7
Period IV	293.7 ± 76.8
Period V	364.3 ± 112.8

been summarized in Table 1. The seven dogs, four males (one neutered) and three females, had a mean weight of 20.1 ± 4.2 kg. Blood volume, estimated as 8.5% of the body weight, equaled 1,704 ± 326 ml. Individual blood volumes calculated for each dog were used to determine the maximum percent total blood volume shed during the experiment. The maximum percent shed blood volume averaged 65.5 ± 11.3% of the calculated total blood volume. Maximum blood volume loss was 5.7 ± 1.1% of the body weight. It required approximately 40-45 minutes to obtain the experimental objective of 40 mm Hg mean arterial blood pressure (MABP). Reuptake of 40% of the shed blood volume occurred over an average of 170 minutes. The post-reinfusion and terminal state measurements were at subsequent average intervals of 71 minutes.

Results of the hemorrhagic shock experiments have been summarized in Tables 2 and 3. The hemorrhagic period was defined by the reduction of MABP to levels consistent with the production of shock. Throughout the hemorrhagic period, MABP was maintained at the 40 mm Hg experimental objective. MABP increased to 88 ± 23.1 mm Hg following reinfusion. Restoration of blood volume failed to restore MABP to the preshock level and is indicative of the physiological changes, such as pooling in vascular beds, transudation of fluids, and loss of autonomic tone, which are known to occur following prolonged and severe hypotension. There was a progressive decline in MABP during the post hemorrhagic observation period to 43.7 ± 15.1 mm Hg. This pattern is consistent with a loss of compensatory reflexes widely reported in a terminal state of shock in dogs.

MABP was the controlled variable during the hemorrhagic period. It

Table 2. Results of the hemorrhagic shock experiments

Parameter	Sample period I	II	III	IV	V
Number (n)	7	7	7	7	7
Systolic blood pressure (mm Hg)	159.2[a,b] ± 24.8	60.0 ± 9.7	60.0 ± 12.1	115.0[c] ± 23.6	66.7 ± 21.3
Diastolic blood pressure (mm Hg)	119.9[b] ± 13.3	31.4 ± 6.5	28.4 ± 3.7	76.9[c] ± 24.7	33.4 ± 14.6
Mean arterial blood pressure (mm Hg)	133.6[b] ± 15.7	41.0 ± 6.0	38.1 ± 4.5	88.7[c] ± 23.1	43.7 ± 15.1
Pulse pressure (mm Hg)	39.4[b] ± 15.9	26.9[c] ± 10.0	31.2[b,c] ± 12.0	38.1[b] ± 8.8	33.2[b,c] ± 10.8
Central venous pressure (mm Hg)	1.2[b] ± 4.1	- 1.9 ± 2.4	- 2.3 ± 1.9	- 1.5 ± 1.2	- 1.2 ± 1.8
Heart rate (beats/minute)	133.6[b] ± 24.2	139.3[b,c] ± 36.7	176.3[c] ± 32.9	151.9[b,c] ± 41.6	153.0[b,c] ± 58.4
Respiratory rate (breaths/minute)	14.5 ± 9.8	27.1 ± 9.4	20.4 ± 5.4	19.7 ± 7.5	22.3[d] ± 9.5
Packed cell volume (%)	40.7[b] ± 4.6	49.2[c] ± 5.1	56.3 ± 4.7	56.2 ± 6.3	56.8 ± 5.0

Red blood count (cells/mm^3)	5.7[b] ± 0.9	6.6 ± 0.8	7.7 ± 0.9	8.1 ± 1.0	8.5 ± 1.8
Hemoglobin concentration (g/100 ml)	13.2 ± 1.8	15.4 ± 1.3	17.9 ± 1.3	17.7 ± 2.4	17.5[d] ± 2.4
Mean corpuscular volume ($microns^3$)	71.8 ± 5.3	74.0 ± 6.7	73.6 ± 6.7	70.1 ± 6.2	70.6[d] ± 11.5
Mean corpuscular hemoglobin (μμg)	23.3 ± 2.0	23.5 ± 2.6	23.5 ± 2.4	22.2 ± 3.4	22.4 ± 4.9
Mean corpuscular hemoglobin concentration (%)	32.5 ± 2.1	31.6 ± 3.6	31.8 ± 2.0	31.8 ± 5.1	30.8 ± 2.8
Plasma protein conc. (g/100 ml)	6.2 ± 0.7	5.3[b] ± 0.6	6.1 ± 1.0	6.3 ± 1.0	5.9 ± 1.0
Plasma osmolarity (mOsm/kg)	299.7[b] ± 7.1	309.3[c,d] ± 9.2	322.7[d,e] ± 14.7	318.8[d,e,f] ± 12.2	313.4[c,e] ± 6.5

[a] All values expressed as mean ± S.D.

[b,c,e,f] Means with the same letter or no letter are not statistically different; t-test for significance; ($p<0.05$). This method for expression of statistical significance is different from that used in all other tables.

[d] Number (n) = 5.

Table 3. Results of the hemorrhagic shock experiments: pH and blood gas parameters

Parameter	Period I	Period II	Period III	Period IV	Period V
pHa	7.428[a,b] ± 0.076	7.363[b,c] ± 0.103	7.091[d] ± 0.179	7.131[d,e] ± 0.247	7.263[c,e] ± 0.192
$PaCO_2$ (mm Hg)	33.0[b] ± 5.8	19.8 ± 4.4	19.9 ± 16.1	20.4 ± 9.2	19.5 ± 8.6
HCO_2 (meq/l)	21.1 ± 2.1	11.3 ± 4.0	6.1 ± 6.7	6.9 ± 3.2	9.5 ± 7.3
Base excess/ deficit (meq/l)	-1.7[b] ± 2.7	-11.2[c] ± 5.6	-25.3[d] ± 10.5	-20.5[d,e] ± 8.4	-16.5[e] ± 6.3
PaO_2 (mm Hg)	108.1[b] ± 19.3	109.1[b] ± 25.6	125.3[c] ± 16.7	119.8[b,c] ± 14.3	114.1[b,c] ± 16.9
PvO_2 (mm Hg)	44.6[b] ± 5.2	20.2[c] ± 9.8	55.2[b] ± 16.9	42.8[b,d] ± 16.0	30.1[c,d] ± 4.2
A-V O_2 difference (ml/100 ml)	3.6[b] ± 1.5	14.5[c] ± 5.1	8.0[d] ± 3.0	10.6[c,d] ± 5.1	12.4[c] ± 4.0
Number (n)	7	7	7	7	7

[a] All values expressed as mean ± S.D.
[b,c,d,e] Means with different letters are significantly different; t-test for significance; ($p<0.05$).

was arithmetically derived from the respective systolic and diastolic blood pressures (MABP = diastolic pressure + 1/3 the systolic-diastolic pressure difference). Pressures of less than 60 mm Hg, such as observed during the hemorrhagic period, would be expected to compromise perfusion of most of the organ tissues of the body. Pressure was restored to an average of 115 ± 23.6 systolic and 76.9 ± 24.7 mm Hg diastolic upon reinfusion. However, pressure alone would be no guarantee of local blood flow. Pulse pressure was altered significantly only during the early hemorrhage when blood was initially lost and stroke volume reduced. Compensatory responses to restore blood volume, such as fluid shifts and intense vascular constriction no doubt contributed to the normalization of pulse pressure. Central venous pressure (CVP) declined significantly with the loss of circulating blood volume and never returned to control levels.

Hypotension characteristically produces reflex sympathetic stimulation. Although heart rate did tend to increase over time in hemorrhage, no significant changes were noted throughout the experiment. Lack of a more pronounced increase in heart rate could have been caused by early depression of the medullary centers or a relatively high initial sympathetic output produced by a vagolytic action of pentobarbital. Additional explanations for the failure to observe a more marked increase in heart rate may be either loss of the sympathetic limb of the baroreceptor response with a predominance of the vagal tone, as observed by Glaviano and Klouda (1965); an acidosis induced depressed myocardial responsiveness to catecholamines, which has been reported by Darby et al. (1960), Ford et al. (1968), Thrower et al. (1961), and

Wildenthal et al. (1968); or the predominant reflex effect of the stimulated peripheral chemoreceptors on vasomotor activity without a major sympathetic effect on the heart, as reported by Downing et al. (1962) and Downing and Siegel (1963). Respiratory rate was almost doubled in early shock but declined to a level about 35% above control during the duration of the experiments. This may also reflect medullary depression or a decreased respiratory drive concurrent with the reduction in arterial $PaCO_2$.

Many reflexes are normally invoked following hemorrhage. Pressure is primarily restored via sympathetic alteration of vascular capacitance and the intense vasoconstriction due to angiotensin. Volume, however, is restored both by renal mechanisms mediated by aldosterone and antidiuretic hormone and by the translocation of fluid into the vascular compartment as hydrostatic pressure is reduced. Fluid movement appears to be reflected in the significant decrease in plasma protein during early hemorrhage. Oxygen carrying capacity may have been partially restored by contraction of the spleen and other reservoirs for red blood cells in the dog. This could explain the increase in the red blood cell count and packed cell volume in early hemorrhage. The hemorrhaged blood entered and left the reservoir at the bottom which may explain why there was a marked increase in the red blood cell count or hemoconcentration during the duration of the experiment despite the apparent dilution of plasma proteins. Blood was reinfused over the course of the hemorrhage that was becoming more packed with red blood cells as they settled. Hemoglobin concentration paralleled the changes in red blood cell count and packed cell volume. Throughout the experiment, no differences were

noted in either the size or hemoglobin content of an individual cell as evidenced by the values for MCV, MCHC, and MCH.

Oxygen content of the arterial and venous samples was determined from the respective values for hemoglobin concentration, PaO_2, pH, and base excess/deficit. Corrections were made for base excess/deficit, temperature, and pH, as described by Altman and Dittmer (1974). The amount of oxygen extracted from the systemic circulation or the arterial-venous (A-V) oxygen content difference was calculated and is shown in Table 3. Extraction increased dramatically during the early hemorrhage. Less oxygen was extracted later in the hemorrhagic period. This may reflect increased shunting which is typical in severe and prolonged hypotension. Extraction of oxygen increased upon reinfusion of all shed blood, but only the final measurement was significantly greater than that observed in Period III. Oxygen extraction is dependent on flow to tissues, tissue utilization itself, and physical factors, like pH, which favor unloading from the hemoglobin. Restoration of blood volume should have improved perfusion to many of the tissue beds. Therefore, perfusion, utilization, and acidity probably all account for the increased extraction. A pronounced oxygen debt which should have accrued during the hypotension would also need to be paid.

Hemorrhage and a progressive state of shock are almost always accompanied by acid-base changes. A progressive metabolic acidosis was observed during the hemorrhage. Accumulation of fixed or nonvolatile acid in early hemorrhage, as indicated by a base deficit of -11.2 ± 2.1 meq/l, became more exaggerated in late hemorrhage and reached $-23.3 \pm$

4.0 meq/l. This would be predicted with a compromised blood flow and loss of the renal route of excretion during the hypotensive period. Respiratory compensation is suggested by the increase in respiratory rate and concomitant reduction in $PaCO_2$. The respiratory rate did not increase to levels which might be expected under the conditions described. This may indicate a central depression of respiratory drive. Any compensation was inadequate to prevent a further reduction in pH from 7.363 $\pm$ 0.103 early in hemorrhage to 7.091 $\pm$ 0.179 during Period IV.

The physiological status of the dogs was not restored to the initial control status following the reinfusion of all shed blood. It appears that there were significant alterations produced by the hemorrhagic period. Mean arterial blood pressure increased to only 88.7 $\pm$ 23.1 mm Hg upon restoration of the blood volume. Central venous pressure also tended to increase. Heart rate was reflexly decreased, while respiration remained unchanged. Although the packed cell volume, hemoglobin concentraton, and the RBC count did not significantly increase, the RBC count did progressively increase from 7.7 $\pm$ 0.4 in late hemorrhage to 8.5 $\pm$ 0.7 million/mm^3. Neither plasma protein concentration, osmolality, arterial $PaCO_2$, nor PaO_2 were altered. The acidosis was partially corrected to 7.131 $\pm$ 0.247, perhaps due to restoration of the blood buffers and restoration of filtration pressure in the kidneys.

The only significant difference observed between the terminal and post-reinfusion periods was the decrease in arterial blood pressure. This decrease was exponential in about half of the dogs and necessitated

taking early measurements. Regardless of the timing of the samples, the MABP was 43.7 $\pm$ 15.1 mm Hg prior to exsanguination. Central venous pressure was significantly higher only during the control period and no difference was observed between the other periods. The CVP progressively declined to a minimum in late hemorrhage, but then tended to increase in the remainder of the experiments. Increased CVP may suggest a central cardiac impairment.

Some impairment of myocardial function seems apparent from the data. The terminal heart rate, concurrent with a progressive hypotension, did not differ from the post-infusion period where blood pressure was partly restored. Marked elevation or depression of the S-T segments in the electrocardiogram was consistently noted as early as Period III in a majority of the dogs. These alterations were more pronounced and observed in all dogs in the terminal stages. Ectopic foci were especially frequent during the terminal period. The tendency for CVP to increase late in the experiment, although not significant, is also suggestive of central cardiac failure. Myocardial depression would certainly be expected with the acidity, reduced myocardial perfusion pressure, and ischemia which are typical of a shock state.

None of the blood parameters were significantly altered in the terminal period. The RBC count tended to increase which may have been due to either a further increase in circulating cells or a loss of vascular fluid. No change in the ratio of cell numbers per fluid volume occurred, however, as evidenced by the constant PCV over the last three periods. Oxygenation of blood did not appear to have been impaired throughout the experiment, as the PaO_2 was consistently in excess of 100

mm Hg.

Discussion

Physiological response to shock *in vivo* involves a complex interaction of many variables acting independently, simultaneously and either cooperatively or antagonistically to one another. Examination of these responses is difficult, at least, in the intact dog and would require equipment and methods beyond the scope of this study. These experiments were conducted to obtain plasma from dogs in a progressive irreversible stage of shock. The data collected are consistent with the patterns reported by Wiggers (1950, 1956), Crowell and Guyton, Rothe and Selkurt (1964), and Bond et al. (1977). Those studies were specifically designed to examine hemorrhagic shock.

It is possible to only speculate whether central cardiac failure was a factor in the loss of compensatory function without a measurement of cardiac output. However, severe metabolic acidosis, the S-T segment alterations in the electrocardiogram which are indicative of myocardial ischemia, and the observed arrythmias late in the experiments are presumptive evidence of some cardiac impairment. Normal canine cardiac function has been shown to be relatively resistant to severe lactic acidosis by Linden and Norman (1969), Downing et al. (1969) and Rocamore and Downing (1969). An effect of acidosis on left ventricular function has been observed by Downing et al. (1966a, 1966b, 1969) when either hypoxia or adrenergic failure was present, but generally not until the acidosis reached a pH of 6.8 - 7.0. A comparable pH in late hemorrhage and an apparently unresponsive heart were observed in this study. Ng et al. (1967), Linden and Norman (1969), and Downing et al. (1971) have

reported that acidosis potentiates vagal slowing of the heart and this may partially explain the lower heart rates late in the experiments. Downing and Siegel (1963) have observed that severe hypotension causes accumulation of metabolites and hypoxia in the peripheral tissues which contributes to a reflex sympathetic vasomotor response but not necessarily increased discharge to the heart. Further, dogs in prolonged hypotension experience a loss of baroreceptor control due to diminished responsiveness of the sympathetic efferents, as reported by Glaviano and Klouda (1965). Loss of blood volume will cause a decrease in the cardiac output and systemic arterial pressure which then elicits an increase in peripheral resistance. Severe hemorrhage, as induced in these experiments, is followed over time by a progressive failure of the animals to maintain systemic arterial pressure. Reuptake of blood concurrent with the maintenance of pressure (at MABP of 40 mm Hg) can only occur if more vascular fluid is lost or the relative blood capacity of the animal increases. A net fluid loss would be consistent with the hemoconcentration observed, but the stable plasma protein concentration throughout most of the recording periods is more consistent with increased erythrocyte mobilization and release rather than concentration via fluid loss.

Considerable evidence from the literature has been cited which supports the failure of peripheral circulation as the primary mechanism for the circulating decompensation in progressive shock. Reduced perfusion pressure and increased sympathetic tone to the peripheral tissues contribute to the accumulation of metabolic products, acid, and hypoxia in those local tissues. Blood flow to a vascular bed is

determined by the net effect of autoregulation of blood flow and the autonomic innervation of that bed. Autoregulation of various tissues beds is independent of the autonomic response and tends to restore local perfusion while the autonomics decrease capacitance in order to preserve blood flow to the brain and heart. The amount of local flow varies with the specific vascular beds within a species. Skeletal muscle vasculature is very responsive to local metabolic products and is well dilated due to autoregulation in shock. This increase in the vascular capacity is thought to be the initiating factor in decompensation, as suggested by Bond et al. (1977). Reuptake of the shed blood during the hemorrhagic period would be partly in response to a normal physiological mechanism. Body temperature was maintained with supplemental heat in the present experiment. Therefore, a normal temperature for metabolic activity in skeletal muscle would have been maintained during a phase of shock when temperature normally would fall due to peripheral constriction. Increased metabolic byproducts in hyperthermia have been shown by Wiggers (1950) to contribute to a more rapid reuptake of shed blood and subsequent irreversible collapse of the animals.

Bond et al. (1976), Bond et al. (1977), Lefer (1973), Kobbold and Thal (1963), Rothe and Selkurt (1964), and Selkurt (1970) have all reported that peripheral circulation may also fail due to the release of vasodepressor or humoral substances. Altered vascular responsiveness to circulating catecholamines, as reported by Bond et al. (1979), may also contribute to peripheral collapse. It is the release of humoral factors, like the myocardial depressant factor (MDF) described by Brand and Lefer (1966), that is most pertinent to the subsequent section of

this study. The pancreatic blood flow is especially compromised in severe hypotension, as shown Bor et al. (1980). Pancreatic hypoperfusion has been shown by Ferguson et al. (1972) to be a causative factor in the production and release of MDF.

Methods: Bioassay of the Shock Plasma

Preparation of papillary muscle

Puppies weighing 1.5-3.5 kg were anesthetized with intravenous sodium pentobarbital (approximately 25 mg/kg). When a satisfactory level of anesthesia was obtained, the chest was opened and the heart rapidly excised and immediately placed in a modified Krebs-Henseleit solution. The heart was then transferred to a second container of Krebs-Henseleit solution (21 C) which was gassed with 100% oxygen.

The chambers of the right side of the heart were opened by making incisions along the lateral margins of the free walls and a papillary muscle was then selected. Criteria for a good papillary muscle were length, cross-sectional area, and shape. Although Pinto (1980) has suggested that the ideal muscle should be greater than 10 mm in length and not over 1 mm in diameter, those dimensions are rare in puppies. A typical puppy muscle is 4-6 mm long and usually triangular in shape. Often the muscle is not distinct, but a composite of adjacent muscles fused at the base. Therefore, it was sometimes necessary to split muscles to obtain an acceptable cross-sectional area. This was accomplished with a #11 scalpel blade inserted between branches of the chordae tendineae of a carefully stretched muscle.

Once the muscle had been selected, a stainless steel hooking device attached to one end of a silver chain was placed around the chordae

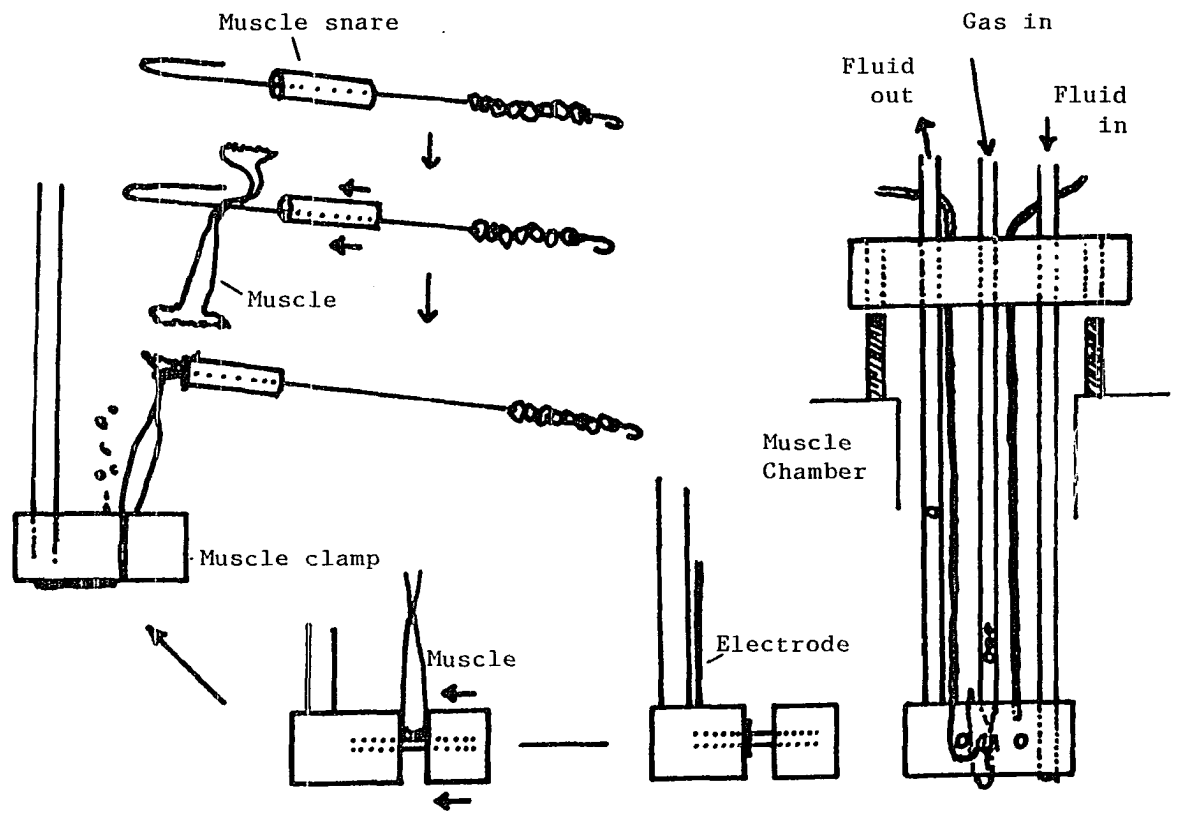


Figure 2. Schematic of the mounting apparatus for the papillary muscle

tendineae and secured, as shown in Figure 2. The chordae tendineae and a small piece of valve were then cut free from the tricuspid valve. A 2-3 mm segment of ventricular wall at the base of the papillary muscle was dissected and the muscle was excised. The base segment was then inserted and clamped in a muscle holder. Care was taken to avoid over stretching the muscle. Muscle, chain, and holder were then transferred into the muscle chamber. The elapsed time from cardiectomy to transfer was less than seven minutes and averaged about 4-6 minutes. Refer to the section Description of bioassay apparatus for details of the bioassay system.

Equilibration of the muscle

A preload weight was initially selected for each muscle based on subjective assessment of length and cross-sectional area. This was usually 0.4 $\pm$ 0.2 g. The preload weight was often increased during the first minutes to carefully stretch the muscle and improve oxygenation and performance. An initial stimulus of 100 volts, 5 msec duration, and 60 hertz was applied. The voltage was decreased to 40 volts as soon as the muscle began contracting and following the stimulus. Frequency of stimulation was slowly decreased to 12 hertz over several minutes once the muscle ceased contracting on its own and completely followed the stimulus. It typically required 1-3 hours at 27 C to equilibrate the muscle. A muscle was deemed equilibrated when no change in isotonic shortening or velocity was observed over 15 minutes. The preload was periodically adjusted up or down during the equilibration procedure towards a maximum isotonic shortening.

Determination of preload

Each muscle was initially equilibrated with a preload weight estimated to be near Lmax. Lmax is the muscle length caused by that weight which when added to the lever will cause a sarcomere alignment which produces a maximum degree of isotonic shortening. A series of known preload weights which usually ranged from 0.04 to 2.0 g were added to the isotonic lever. Sufficient weight, based on a subjective evaluation, was added to the lever and caused stretching of the muscle beyond Lmax, but not in excess so as to damage the muscle. The preload weight was then sequentially decreased as follows. A weight was hung on the lever and the muscle equilibrated for 6-10 contractions (uniform number per given experiment) and then exchanged for a lighter weight. At least 10-12 weights were ordinarily tested with a minimum of three weights stretching the muscle in excess of Lmax. Isotonic contractions were recorded on the polygraph. Recorded amplitudes of the isotonic contractions for each weight were then measured. These data were then fitted (least squares method) to the following formula with a computer. Lmax was determined as the intersection of the ascending and descending limbs of the preload curve as described by the following equations.

EQUATION #1

Ascending limb: $y = a + b (\ln x)$

EQUATION #2

Descending limb: $y = a (e)^{bx}$

The values a and b are estimates of the intercepts and slopes obtained by the fit while e is an irrational number whose value is approximately 2.71828. Each muscle was then adjusted to 90% of Lmax with the

appropriate weight and further equilibrated a minimum of 30 minutes.

Testing of solutions

The respective test solution (either Krebs-Henseleit or plasma) was added to the muscle chamber and equilibrated 30 minutes before measuring responses to various afterloads. Modification of the system for use with plasma is described in the section Description of bioassay system. Krebs-Henseleit solution was always tested first and last for several reasons. Krebs-Henseleit solution functioned as a standard reference throughout this entire study. Changes in the function of isolated tissue with time are normally expected and must be considered in assessing the effects of the various test materials. Such changes can be minimized by proper mounting technique, small cross-sectional area, and maintenance of pH and oxygenation. Further any irreversible effects of the depressant materials on muscle must be differentiated from those changes with time. Periodic measurements with the Krebs-Henseleit solution over the 5-8 hour experiment gave a relative indication of time changes which could be contrasted to changes occurring in the relatively short time span for testing other material or agents. Data obtained during the last treatment with the Krebs-Henseleit solution were used for all comparisons to both control and shock plasma. It should be recognized that this tends to negate changes with time but also tends to underestimate any depression caused by the plasma or other intervention.

Afterloading of muscle

Muscle response to each test solution was evaluated after equilibration by measuring the contractile response to a series of known afterloads. Refer to the section Description of bioassay equipment

for complete details on the operation of the equipment. The basic procedure was as follows. Preload weight was maintained on the isotonic lever and kept the muscle at 90% Lmax. A bar attached to the hydraulic cylinder of a microdrive unit (David Kopf Instrument Inc.) could then be lowered to the lever removing some of the preload weight and cause shortening of the muscle. Known weights (afterload) were added and the bar repositioned so as to reestablish the sarcomere alignment at 90% Lmax as determined by the polygraph recording. After approximately eight contractions, two successive contractile cycles were recorded at 25 mm/sec on the polygraph. The afterload was then removed, the bar completely withdrawn from the lever, and the muscle allowed to equilibrate approximately eight contractions before the next afterload. The initial priority was to locate the maximum load (Po) which just prevented isotonic shortening. All subsequent afterloads tested were of a decreasing magnitude. Number of weights tested ranged from about 10-20. It usually took 20-30 minutes to complete an afterload curve. The response to the preload weight was recorded at the beginning and end of the test and was used as an indication of changes with time and irreversible changes due to the shock or control plasma over the test interval. The smallest afterload weights were generally 0.12, 0.08, and 0.04 g, respectively. It is known that the relationship between load and the other measured parameters is relatively linear over the middle range of afterload weights between preload and Po. Therefore, more data were collected at the extremes where changes are less linear.

Measured parameters and calculations

Measurements made from the recordings are shown schematically in Figure 2 and are defined as follows.

1.) Load (P) = known afterload weight.

2.) Maximum velocity of contraction (Vc) : calculated from the maximum angle drawn as a tangent to the contractile phase of the isotonic contraction (θc).

EQUATION #3

$$Vc = (\tan \theta c) \times (\text{calibration factor in mm/mm}) \times (\text{chart speed in mm/sec})$$

3.) Maximum velocity of relaxation (Vr): calculated from the maximum angle drawn as a tangent to the relaxation phase of the isotonic contraction (θr)

EQUATION #4

$$Vr = (\tan \theta r) \times (\text{calibration factor in mm/mm}) \times (\text{chart speed in mm/sec})$$

4.) Calibration factor: At the conclusion of each experiment the chain attached to the muscle was disconnected from the transducer lever and the lever displacement, calibrated in microns/millimeter of pen deflection, was recorded and converted to millimeter of isotonic shortening/ millimeter pen deflection.

5.) Latency intervals: The intervals from stimulus artifact to various phases of the contractile cycle were measured on the recording with a micrometer and converted to time (distance in mm/ chart speed in mm/sec).

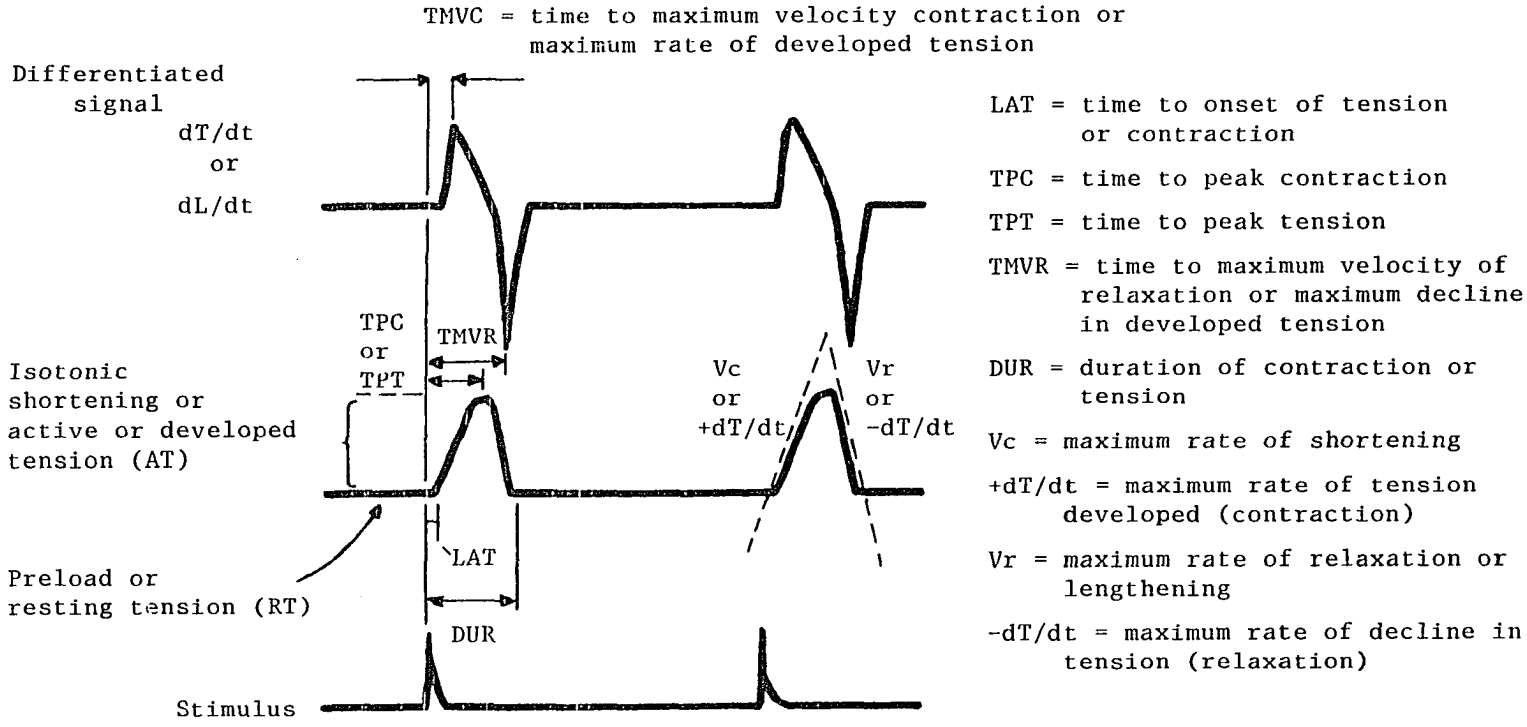


Figure 3. Measured parameters in the papillary muscle bioassay

EQUATION #5

Time = (distance in mm) / (chart speed in mm/sec)

These intervals are defined as follows:

Latency (LAT) = time from stimulus to onset of the contraction.

Time to maximum velocity of contraction (TMVC) = time from stimulus to the peak of the differentiated signal.

Time to peak of contraction (TPC) = time from stimulus to the peak of the isotonic contraction.

Time to maximum velocity of relaxation (TMVR) = time from stimulus to the negative peak of the differentiated signal.

Duration of the contraction (DUR) = time from stimulus to the return of the isotonic contraction tracing to the baseline.

Data were expressed per cross-sectional area where appropriate. All the afterload data for a given treatment were then fitted to a third order polynomial equation (least squares method). Normalization of data between muscles was accomplished by calculating from each polynomial equation the respective values for each parameter which corresponded to ten percent increments of Po.

EQUATION #6

$$y = c + ax + bx^2 + cx^3$$

Measurement of muscle length

A catheotometer was used during the experiment to determine muscle length to the nearest 0.1 mm while the muscle was at 90% Lmax. The most difficult aspect of this measurement was assessing where muscle ended and chordae tendineae began. Visualization was optimized by focusing a light on the muscle. Multiple measurements were made and the values

were generally very consistent.

Measurement of cross-sectional area

At the conclusion of all testing, the muscle holder, chain, and muscle were removed from the chamber. The muscle was transected as it entered the holder with a scalpel. A dissecting microscope was then used to visualize and cut the chordae tendineae from the opposite end of the muscle. The muscle was then blotted and weighed on an analytical balance to the nearest 0.1 mg. This procedure was repeated several times and an average value used for subsequent calculations. Each measurement was made only after the muscle was resubmerged in Krebs-Henseleit solution. Cross-sectional area was determined by the following formula:

EQUATION #7

$$XSA = \frac{(1.065\ mg/mm^3) \times (\text{weight of muscle in mg})}{(\text{muscle length in mm})}$$

The value 1.065 mg/mm^3 is an approximation of the muscle density reported by Park and Driscoll (1982) for canine myocardium.

Description and construction of the bioassay system

The bioassay system consisted of a muscle chamber/water bath, a mounting apparatus for positioning the papillary muscle in the muscle chamber and attaching the muscle to the recording system, a microdrive unit for altering and maintaining muscle length, a recording system, and a peripheral system for circulating fluid in the muscle chamber and oxygenating plasma.

Mounting apparatus for papillary muscle The muscle clamp consisted of two Plexiglas plates connected by three stainless steel tubes, as shown schematically in Figure 3. Gas for oxygenation of the chamber fluid was passed through one tube while the other two functioned as ports for circulating the chamber fluid via a circulating pump. The top plate mounted to the top of the water bath/ muscle bath system and maintained the muscle clamp in a fixed position. The lower plate was divided into two sections. One was permanently attached to the stainless steel tubes while the second formed the actual clamp. It could slide over a pair of stainless steel tubes embedded in the other half of the plate. When the clamp was closed it would securely hold the wall end of the dissected papillary muscle. A stimulating electrode was embedded into the fixed half of the clamp in intimate contact with the muscle. A second electrode was mounted adjacent to the three stainless steel tubes and parallel to the muscle.

A snare device was used to connect the other end of the muscle to the transducer. It was constructed from a stainless steel rod bent to form a snare, as illustrated in Figure 3. A small piece of stainless steel tubing was slipped down over the rod and was utilized to close and securely lock the snare around the chordae tendineae. The other end of the rod was attached to a silver chain which connected to the lever mounted on the transducer.

The main gas tube was divided at the base and connected to three 25 gauge tubes projecting from the clamp near the base of the muscle. This increased the oxygenation of the fluid by increasing the number of smaller gas bubbles and also should have contributed to mixing of the

chamber fluid. Oxygen tensions in the circulating fluid measured in excess of 500 mm Hg. One of the vertical tubes for circulating fluid was partially transected at a level near the top of the muscle bath. This enabled circulation of fluid from bottom to top of the chamber and also promoted good mixing of the fluid.

Chamber system The double chambered system consisted of a muscle bath or chamber surrounded by a water bath for maintenance of constant temperature. This device is schematically presented in Figure 4. The water bath or outer chamber was formed by sandwiching a Pyrex glass staining jar between two Plexiglas plates. A rubber gasket was placed between the plates and the glass and the chamber was sealed by a series of bolts which compressed the plates around the jar. Water was heated to maintain the bath temperature at 37 C and was circulated between a Haake constant temperature water bath and the outer chamber through ports installed in the top plate. Equilibration of the water bath, initially at room temperature, required no more than 30 minutes. Test solutions were prewarmed to 37 C prior to being exchanged. However, even solutions at room temperature would warm to 37 C within 1-2 minutes when added to the testing chamber. Temperature in the water bath remained constant over the duration of any experiment.

The muscle chamber itself consisted of a glass vial which was fitted beneath a hole in the center of the top plate and into a recessed area on the inner aspect of the plate. It was sealed to the top plate by compression created when a small Plexiglas plate positioned beneath the vial in the chamber was bolted to the top plate. Vials were periodically cleaned with acid and resiliconized. The chamber volume

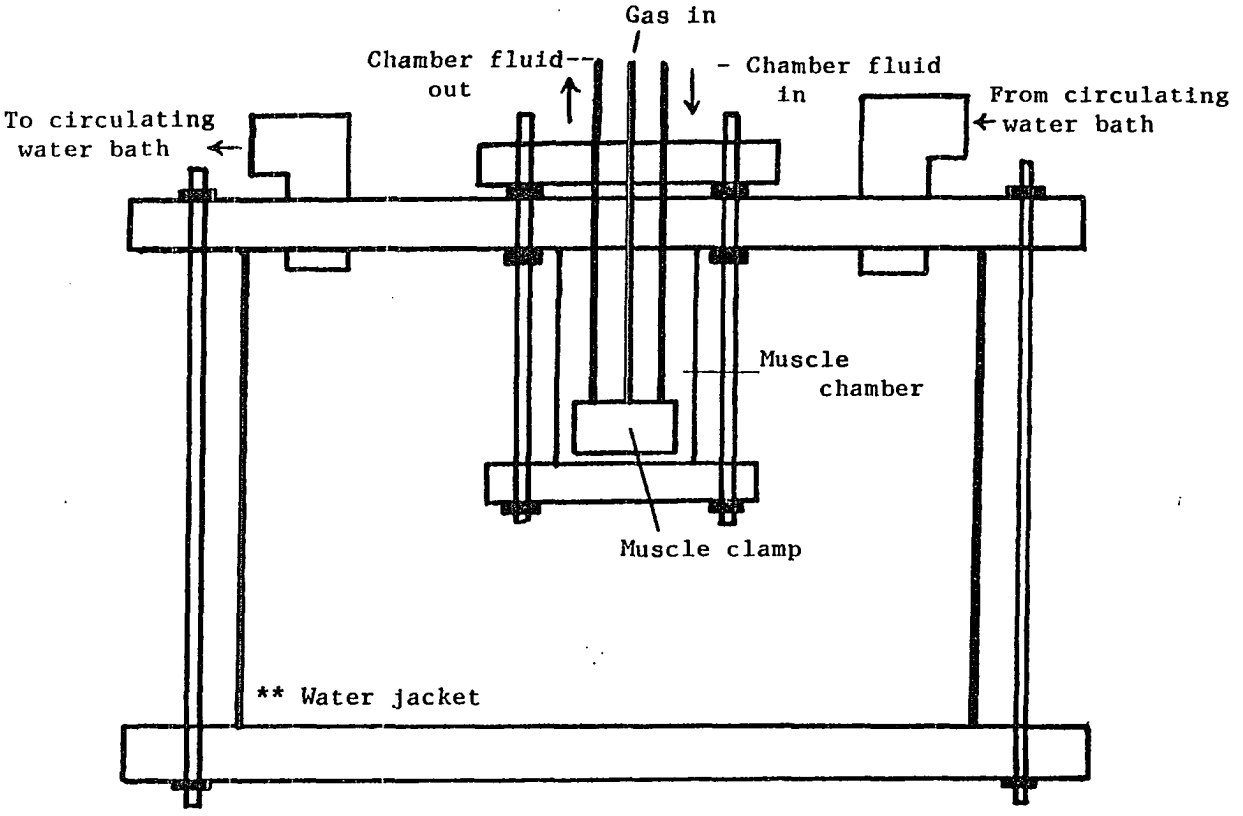


Figure 4. Schematic diagram of the chamber system for bioassaying papillary muscle.

was normally between 8-10 ml with the muscle clamp mounted. However, the same volume was always used on a given day when testing. This system was adequate to permit visualization of the muscles from the side for gross observation and measurement of muscle length with a catheotometer. The intact chamber system was mounted on a track system. It could be moved laterally by a ratchet device to align the muscle with the transducer and then securely locked to prohibit movement.

Fluid was recirculated through the chamber by a pump located external to the chamber. Emptying the chamber could be accomplished by disconnecting the tubing connecting the chamber with the pump and pumping the fluid out. A combination pH probe was mounted into a "T" device inserted into the external fluid circuit. The combination of a flat bottom probe and the design of the device helped to minimize the volume of fluid in the "T" device. An Orion Research Model 601A digital pH meter was used to continuously monitor pH throughout each experiment.

A hydraulic cylinder driven by a microdrive unit (David Kopf Instruments Inc.) with a rigid rod attached was positioned over the end of the isotonic muscle lever and was used as a stop to limit the movement of the isotonic muscle lever. Extension of the cylinder in micron units permitted very accurate positioning of the lever system. During an afterload measurement the lever could be lowered, causing shortening of the resting muscle length, and a selected afterload weight could then be added to the opposite end of the lever. The muscle could then be returned to the resting length at 90% Lmax by withdrawing the cylinder until the appropriate length, as determined by the polygraph recording, was reached. Known changes in the position of the muscle

lever caused by raising and lowering the cylinder were used to calibrate the polygraph recording for muscle length changes.

Muscle shortening was measured with a isotonic muscle transducer (Gould Inc.) and recorded on a Beckman R-611 polygraph. Output of the channel recording change in muscle length was differentiated and the change in muscle length per time was recorded as a an indicator of velocity. The differentiator was designed and built by Dr. Wang Tang, Electrical Engineering Department, Iowa State University. Delivery of the electrical stimulus by a Grass SD9 stimulator to the muscle through silver electrodes was also monitored on the polygraph. Continuous recordings of all parameters were made throughout each experiment.

Diffuser system A diffusion system was designed to maintain adequate oxygen tensions yet eliminate the foaming when whole plasma was added to the muscle chamber. Schematics of the system are shown in Figure 5.

The chamber was constructed from two plates of Plexiglas (0.79 x 3.81 x 45.4 cm). Specific measurements are given in the schematics. A groove was made on the central portion of one plate. The plates were then bolted together to form a sealed chamber. A series of seven stainless steel tubes were sealed in holes drilled into each end of the chamber. Seven lengths of a thin-walled silastic tubing (Ronsil sialatube, Ronsil Rubber Products, Division Rodhelm Reiss, Inc., Bell Mead, N.J.) with an I.D. of 0.889 mm, and a wall thickness of 0.254 mm were fitted into the groove and onto the tubing ends extending into the chamber. Tubes were placed in series by attaching a short length of polyvinyl tubing to the appropriate stainless steel tubes extending

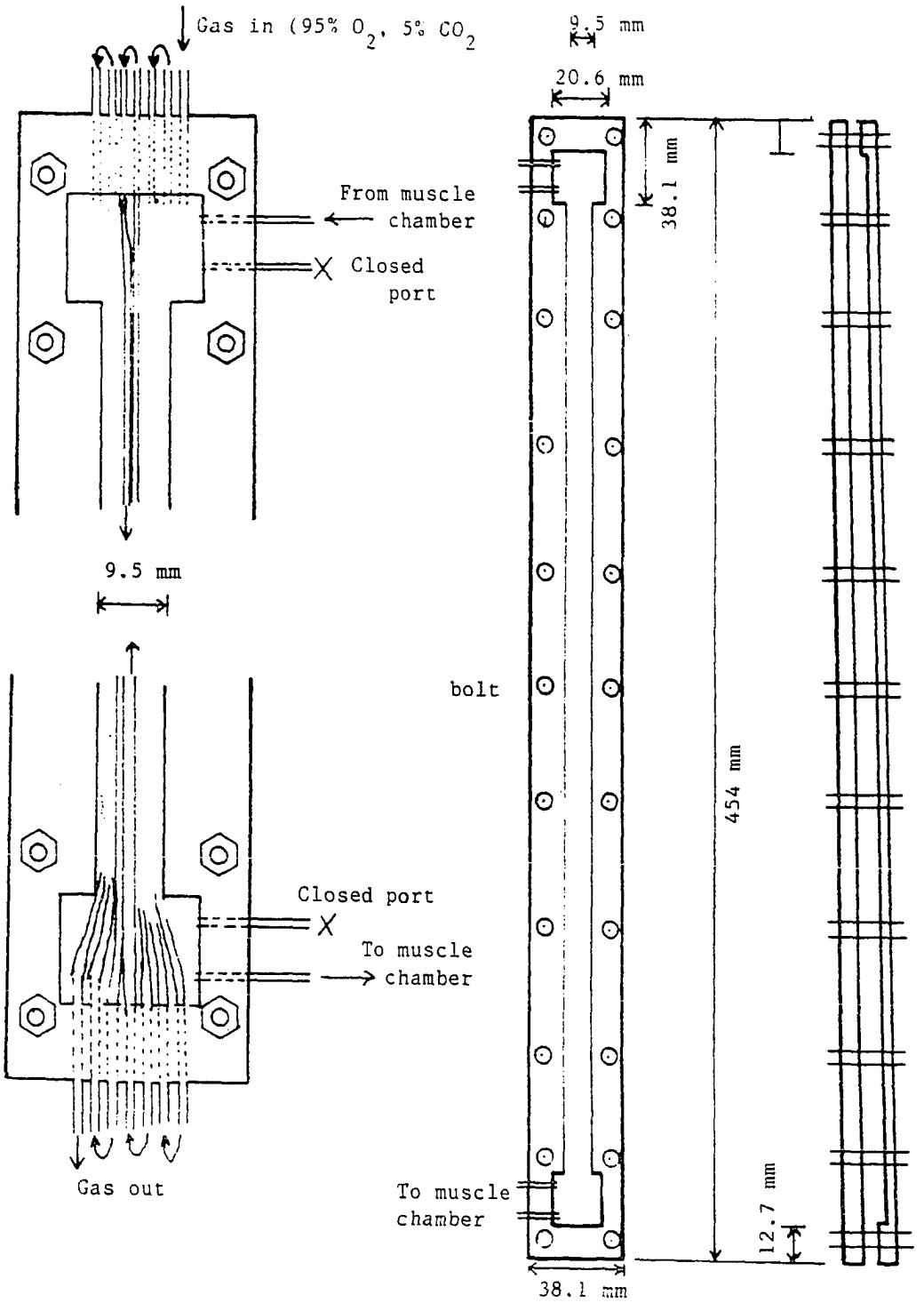


Figure 5. Schematic diagram of the diffuser system for assaying plasma

outside of the chamber. A single tube on either end functioned, respectively, as the inlet for gas (95% O_2, 5% CO_2) and an outlet. The gas was saturated with water vapor prior to entering the chamber. Two additional stainless steel tubes were inserted into the chamber on each end of the chamber as inlet and outlet ports for the circulated fluid (plasma or K-H). Only one port on each end was normally used. The long continuous series of silastic tubing created a large surface area for optimum diffusion of oxygen out into the fluid circulated through the chamber around the silastic tubing. Fluid was circulated by either the circulating pump in the assay system or an auxiliary pump used only when the fluid was being equilibrated prior to connecting into the system. Approximately 1.5 ml of fluid was accommodated inside the chamber which created a surface area to volume ratio sufficient to maintain the PO_2 in the fluid in excess of 500 mm Hg.

Results and Discussion

Muscle parameters and composition of the test solutions

The characteristics of the papillary muscles used for bioassay and the concentration of selected electrolytes in the test solutions are summarized in Table 4. Although seven shock experiments were conducted, only four bioassays of the whole plasma harvested from those experiments could be completed. The remaining experiments could not be completed the same day because of either irreparable damage to the muscle without suitable replacement or failure to obtain muscles of sufficient quality from the available animals which were stable enough to conduct the tests. Any attempt to store plasma for later assay would have been unacceptable as the composition of the plasma may have been altered over

time due to proteolysis, precipitation of proteins, or alterations in calcium concentration or osmolarity. Resolution of the presence of an endogenous depression may also have been obscured by the degradation of the molecule during storage. The muscles which were tested were some of the better quality muscles utilized throughout this entire project. At least 8-10 hours minimum were required to complete the assays which included approximately one hour for each solution. Despite the duration, there was only a ten percent difference between K-H1 and K-H2 in maximum isotonic load (Po) and approximately twenty percent change in velocity of contraction over the time period from the beginning of testing to completion. There was a time lag of 0.5 - 1.5 hours between K-H1 and the next solution tested.

There were significant differences in the composition of the test solutions, as shown in Table 4. Sodium concentration, however, was remarkably constant in all solutions and did not differ. Potassium is normally lost from the intracellular site when there is a loss of cellular integrity, as in shock.

The potassium concentration in the shock plasma was significantly increased by 60% and 70% relative to the Krebs-Henseleit solution and control plasma. Two papillary muscles were used in pilot experiments to compare a Krebs-Henseleit solution containing 7.5 meq/l of potassium with the standard Krebs-Henseleit. No discernible effects on the same measured parameters were observed.

A significantly lower calcium concentration was also measured in the shock plasma relative to both K-H and control. The reduction amounted to 13% and 16%, respectively. A linear relationship between

Table 4. Summary of muscle characteristics and composition of the tested solutions

Muscle characteristics :

Muscles: n = 4
Length = 6.4 ± 0.5 mm [a]
Cross-sectional area = 1.41 ± 0.45 mm^2

Selected electrolyte composition of the test solutions :

Parameter	Krebs-Henseleit	Control plasma	Shock plasma
Sodium (meq/l)	143.8 ± 0.5	143.3 ± 2.2	144.5 ± 1.3
Potassium (meq/l)	4.5 ± 0.3	4.3 ± 0.4	7.3[b,c] ± 2.1
Calcium (meq/l)	2.21 ± 0.08	2.29[b] ± 0.11	1.92[b,c] ± 0.17
Osmolarity (mOsm/kg)	290.0 ± 4.3	297.0[b] ± 4.4	313.5[c] ± 8.7

[a] All values expressed as mean ± S.D.
[b] Significantly different from Krebs-Henseleit; ($p<0.05$).
[c] Significantly different from Control plasma; ($p<0.05$).

isotonic shortening of canine papillary muscles and calcium concentration has been reported by Hembrough et al. (1978). Wangensteen et al. (1973) and Goldfarb et al. (1979) have observed a similar relationship between isometric developed tension and calcium with feline papillary muscles. A 1% change in calcium concentration produces approximately a 1% change or less in tension or shortening. This relationship was also observed in two preliminary experiments in which the isotonic responses of canine papillary muscles were recorded at various calcium concentrations. In one of those experiments, the relationship between calcium concentration and maximum load (Po) was expressed by the formula $Y = -0.11 + 12X$ ($r = 0.996$), where Y = the calcium concentration in g/100 ml and X = the maximum load (Po) in grams. Calcium versus velocity of contraction were related by the formula $Y = 0.17 + 0.37\ Z$ ($r = 0.996$), where Y = the calcium concentration in g/100 ml and Z = the maximum velocity of contraction in mm/sec.

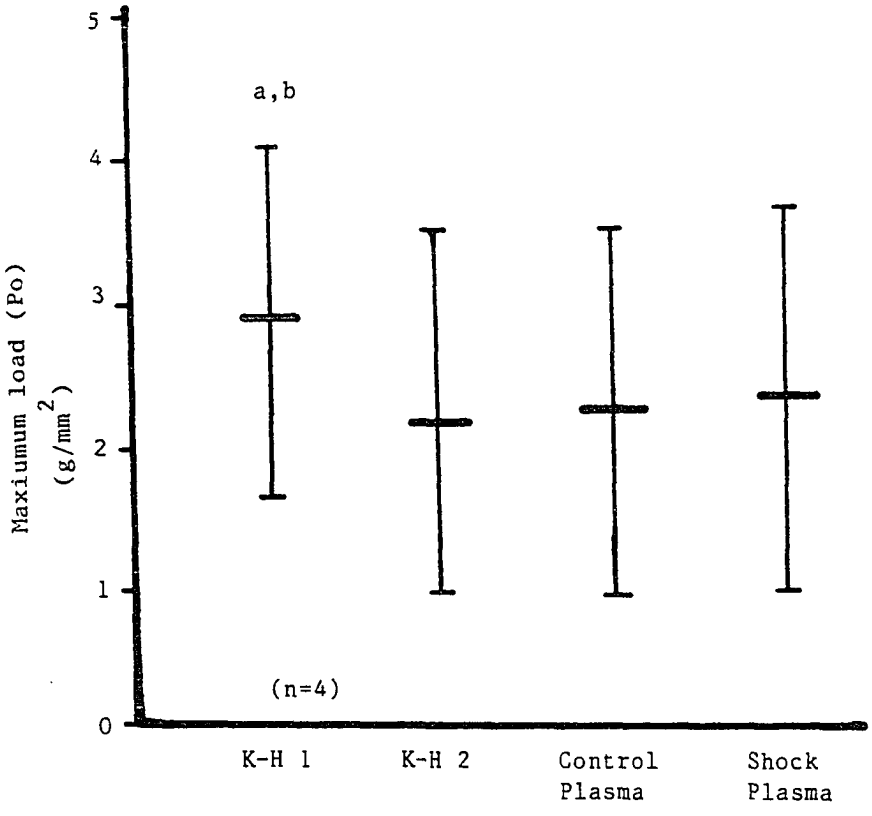
Osmolarity differed significantly between the three solutions. However, osmolarities as high as 350 mOsm/kg have not produced changes in muscle function when examined previously. This also is consistent with the data of Lefer and Inge (1973) and Goldfarb et al. (1979).

Both plasmas were tested without modification of electrolyte or osmolar composition. The contribution of these electrolyte and osmolarity changes, in lieu of the evidence cited, may only be minor. However, all shock plasmas were very acidic and required addition of more 0.1 N NaOH in order to buffer them to pH 7.4. Control plasma required less buffering to adjust pH. Initial buffering to 7.4 was

transient and the pH needed constant adjustment throughout the experiment. The pH was constantly monitored and adjusted which may have minimized any effect of acidosis on the muscle. The pH of the chamber fluid was not allowed to decrease to less than 7.3. Continuous monitoring of pH in previous reported studies was not done. Therefore, an acidic condition may have been present during those bioassays despite initial adjustment of pH. Siegel and Downing (1970) have observed that acidosis does not affect isolated tissue function until a pH of 6.8 or less was present or at higher pH when hypoxia was present. No effect of pH as low as 6.6-6.7 has been observed in some preliminary studies in which 1 N HCl was added to the muscle bath to reduce pH. However, an occasional muscle would respond to a few drops of 0.1 N HCl but then produced a control recording within several beats. Little effect of pH on muscle function over the range in this study would be anticipated as observed in previous studies by Goldfarb et al. (1979).

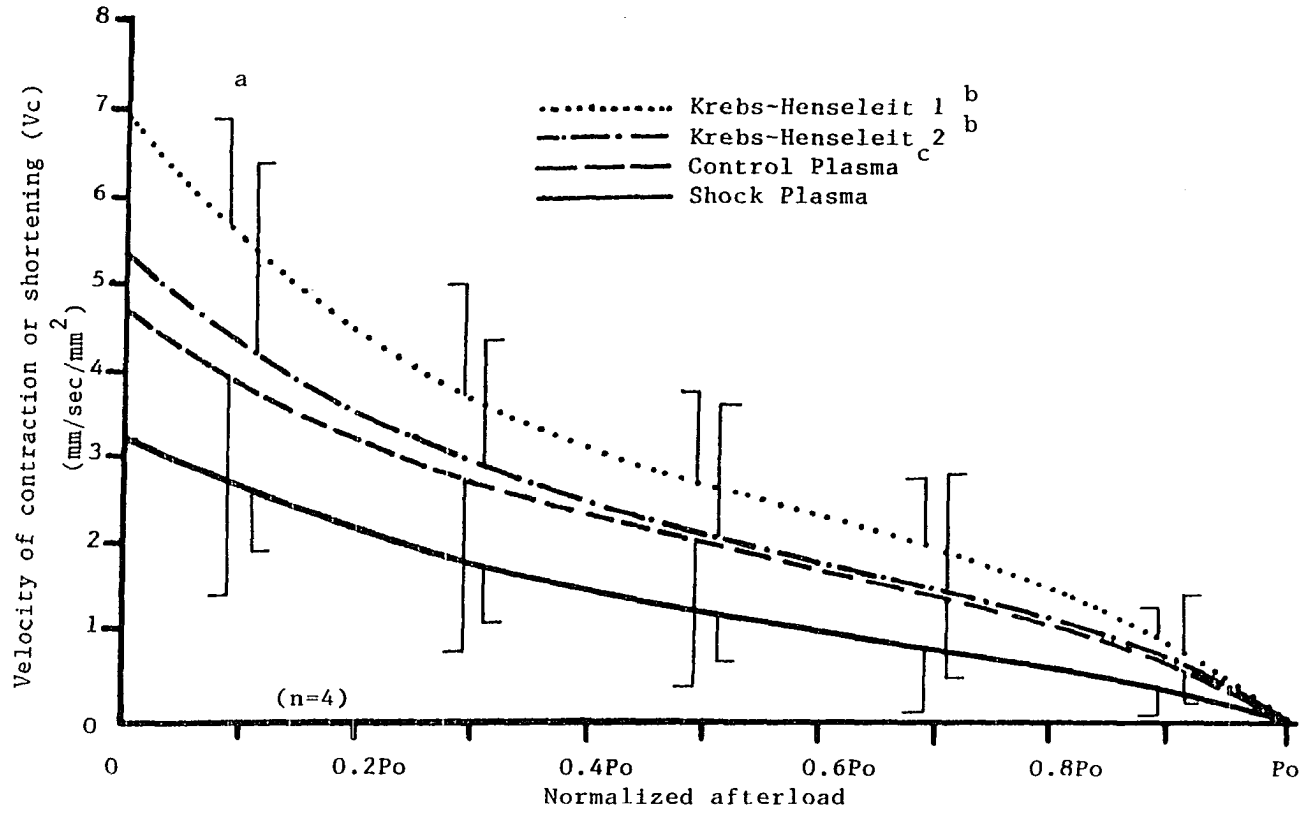
Results of the bioassays with isotonically contracting canine papillary muscles have been summarized in Figures 6-14. All data have been expressed as mean $\pm$ S.D. A Student t-test was utilized to determine statistical significance. These experiments demonstrate that shock plasma may contain a depressant substance.

Mean maximum loads (Po) recorded for each solution are shown in Figure 6. No differences were found between any of the solutions. Maximum load for the shock plasma was actually slightly greater than observed for either control plasma or K-H2. The activity of MDF has been defined in units equivalent to a 1% decrease in the developed isometric tension by Brand and Lefer (1966). If peak developed tension



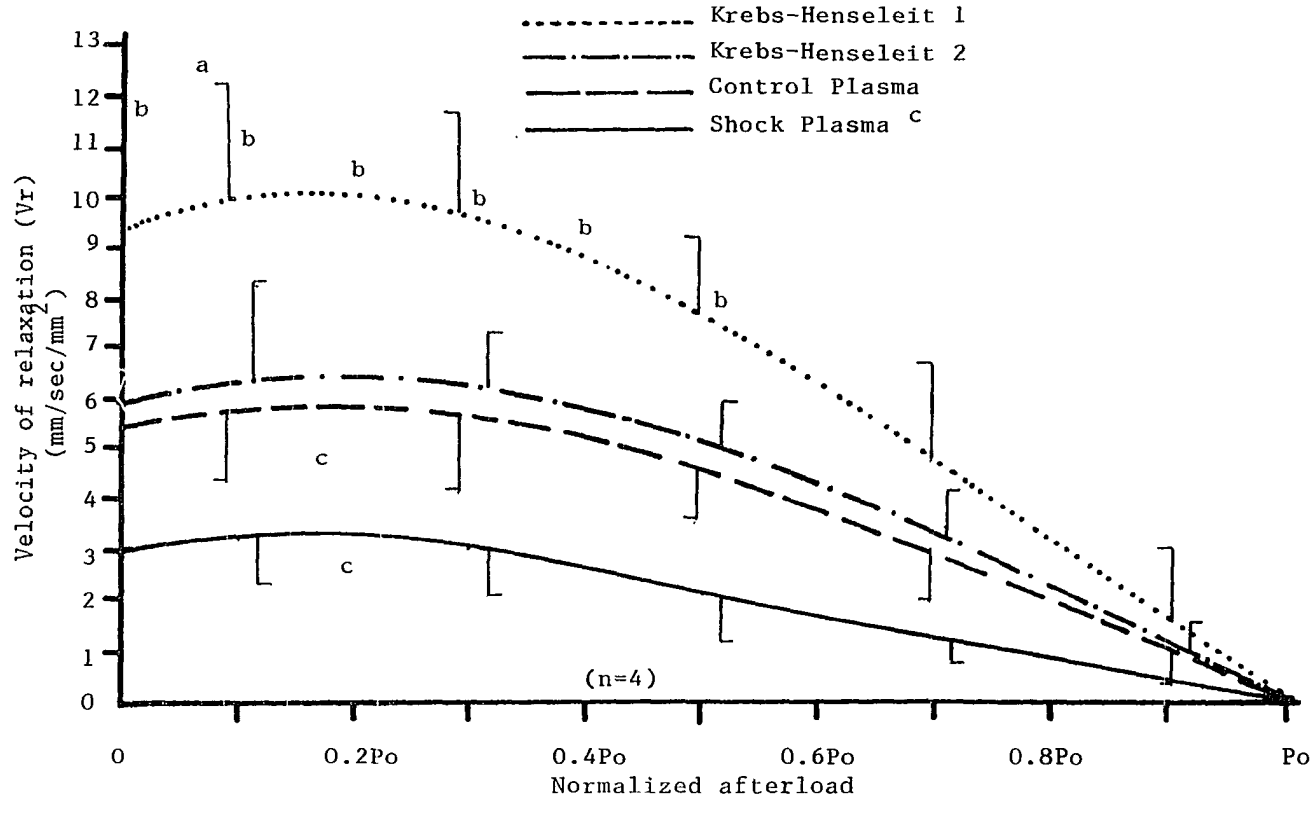
[a] All values expressed as mean ± S.D.
[b] No significant differences; t-test for significance.

Figure 6. Maximum load (Po) recorded for each of the test solutions



a Mean ± S.D. at corresponding afterloads; bars staggered for clarity.
b Significantly different from shock plasma at all afterloads; ($p<0.05$).
c Significantly different from shock plasma at 0, 0.8-0.9Po; ($p<0.05$).

Figure 7. Velocity of contraction or shortening versus afterload for all test solutions



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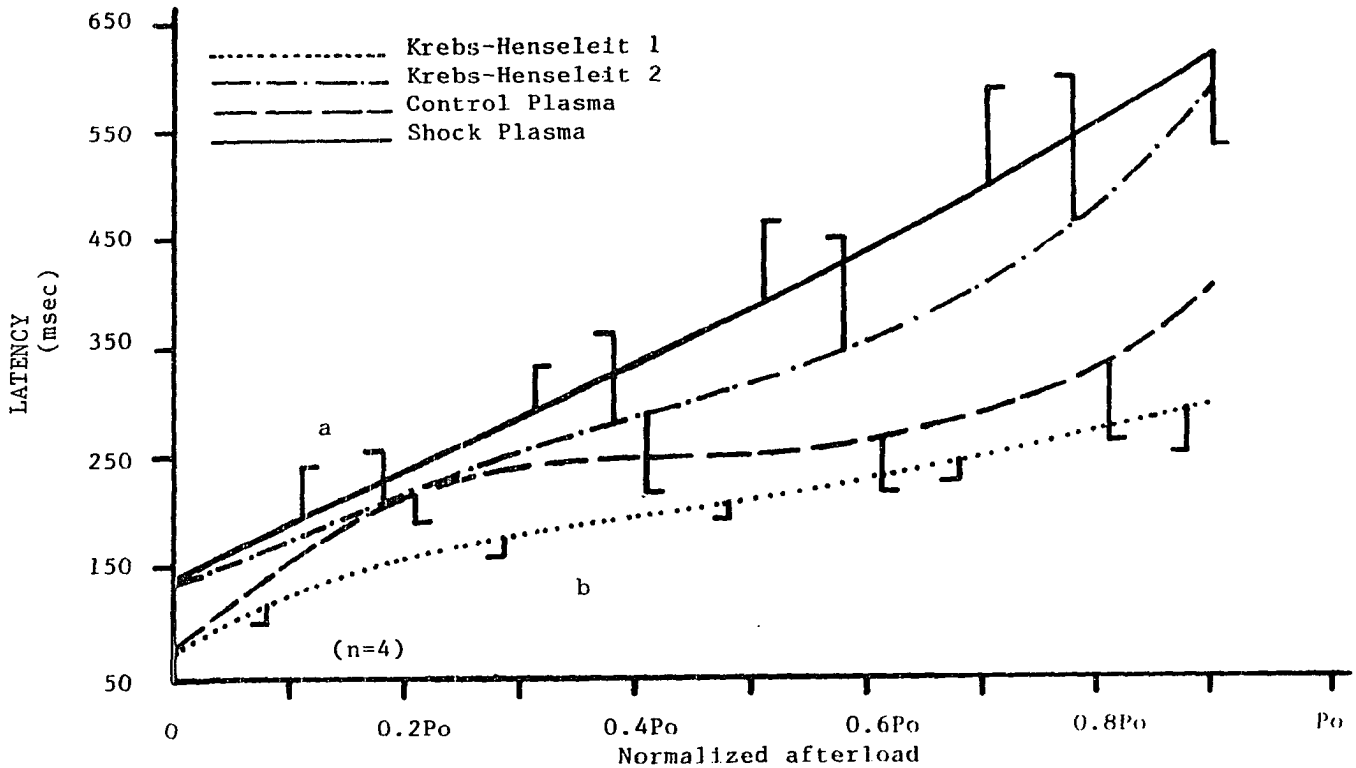
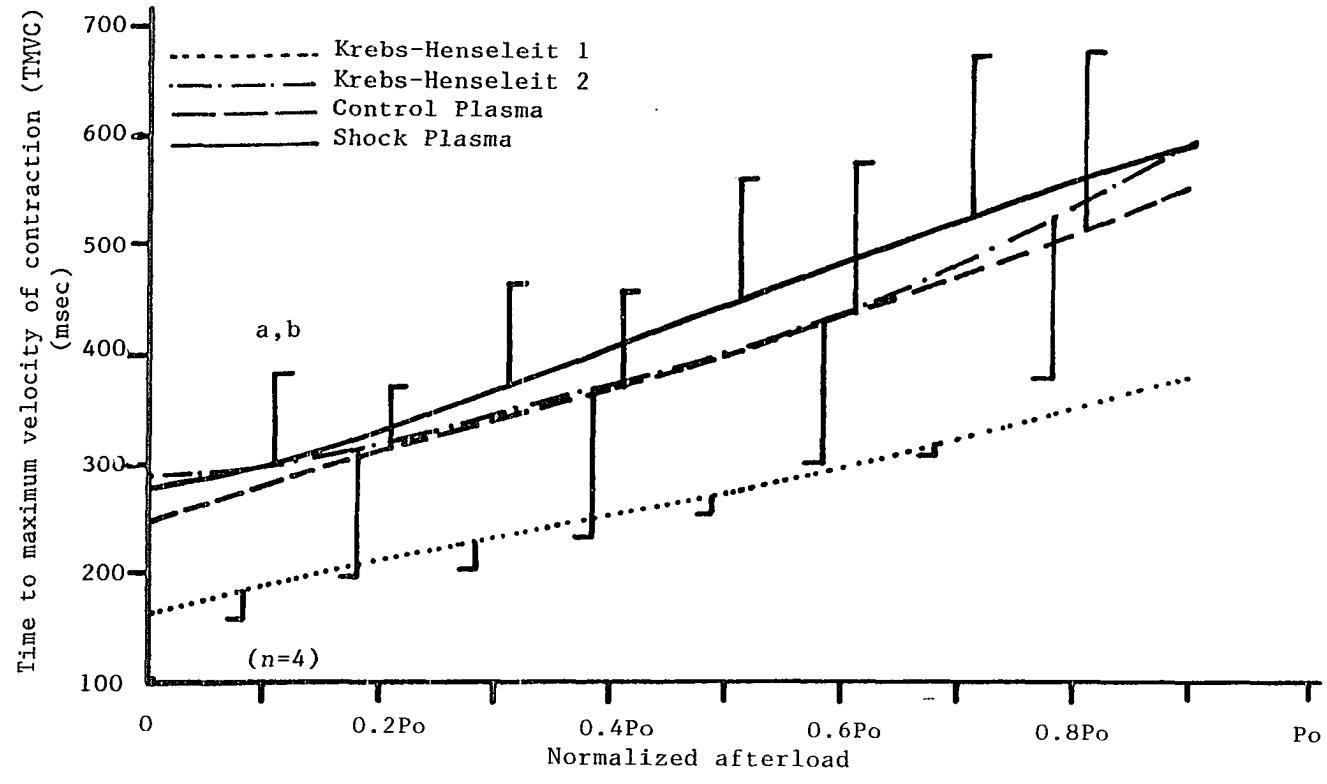
[a] Mean ± S.D. at corresponding afterload; bars staggered for clarity.
[b] Significantly different from K-H2 and Control plasma between 0-0.5Po; ($p<0.05$).
[c] Significantly different from all other solutions at all afterloads; ($p<0.05$).

Figure 8. Velocity of relaxation versus afterload for all test solutions



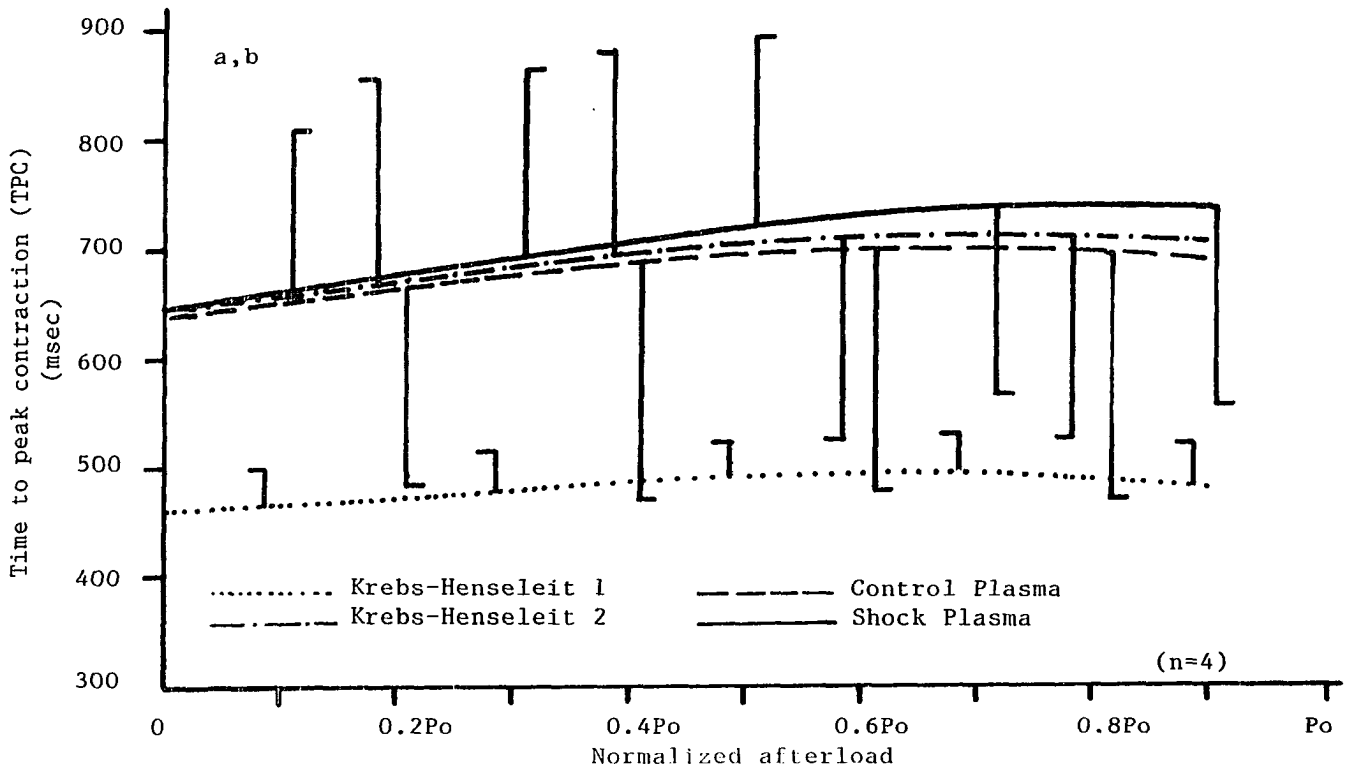
[a] Mean ± S.D. at corresponding afterload; bars staggered for clarity.
[b] No significant differences; t-test for significance.

Figure 9. Effects of the test solutions on latency (LAT)



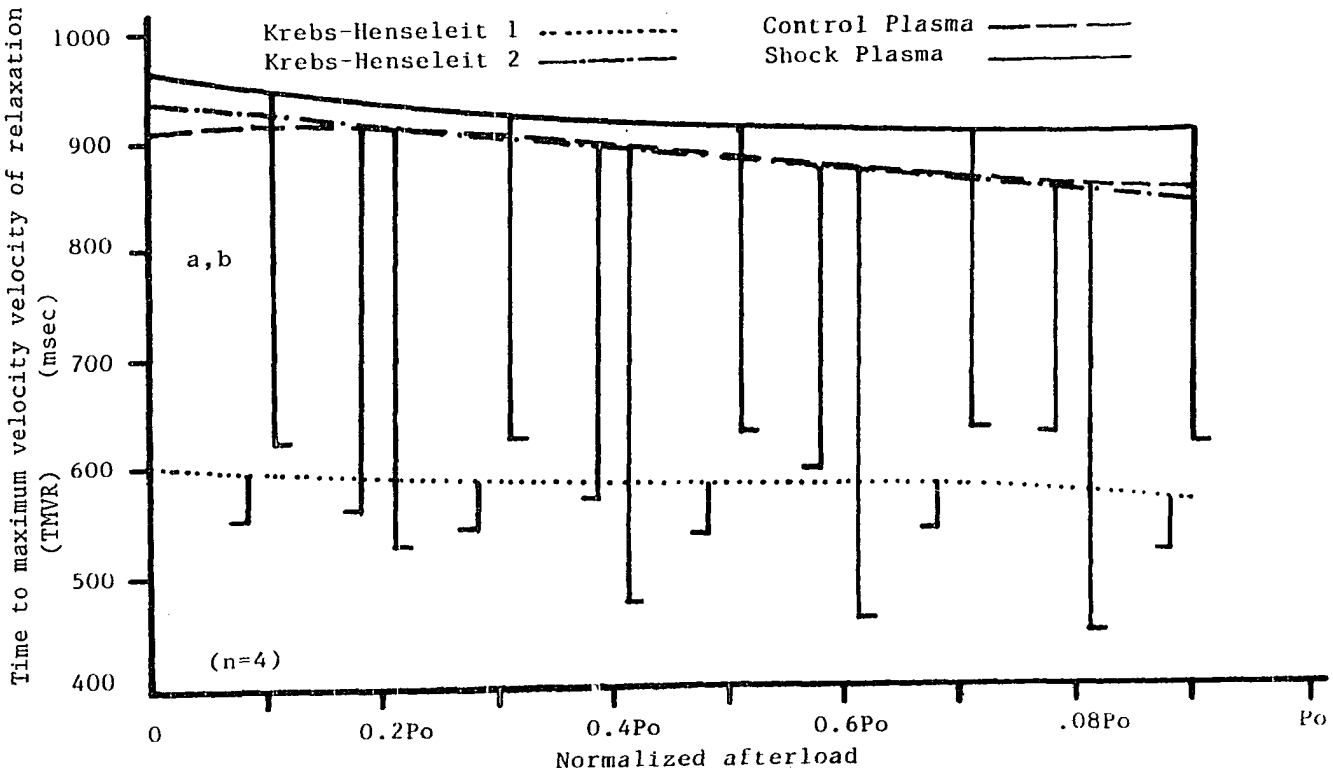
[a] Mean ± S.D. at corresponding afterload; bars staggered for clarity.
[b] No significant differences; t-test for significance.

Figure 10. Effects of the test solutions on time to maximum velocity of contraction (TMVC)



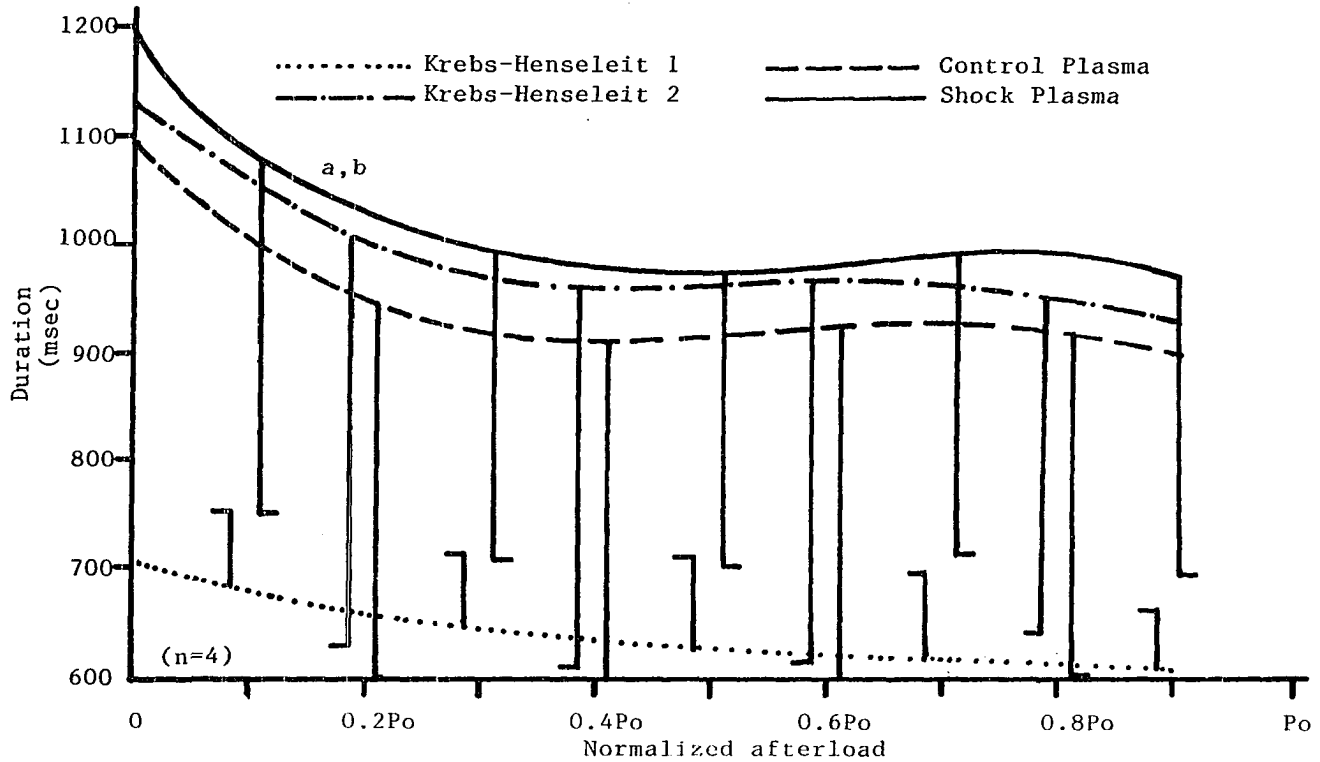
[a] Mean ± S.D. at corresponding afterload; bars staggered for clarity.
[b] No significant differences; t-test for significance.

Figure 11. Effects of the test solutions on time to peak of contraction (TPC)



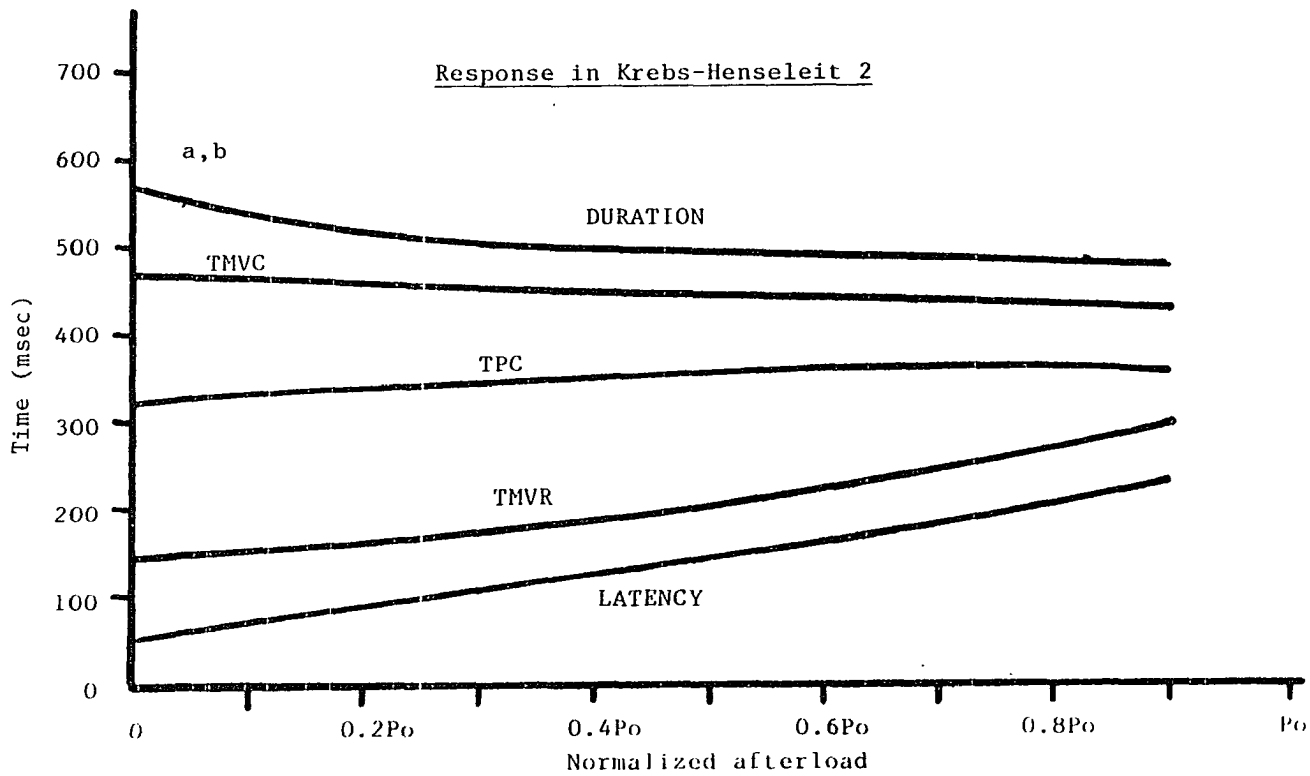
[a] Mean ± S.D. at corresponding afterload; bars staggered for clarity.
[b] No significant differences; t-test for significance.

Figure 12. Effects of the test solutions on time to maximum velocity of relaxation (TMVR)



[a] Mean ± S.D. at corresponding afterload; bars staggered for clarity.
[b] No significant differences; t-test for significance.

Figure 13. Effects of the test solutions on duration of contraction (DUR)



[a] Mean values only.
[b] No statistics applied.

Figure 14. Example of the typical relationship between the various time measurements over the range of afterloads tested

was the only criterion used, then it would be inferred that no depressant action was present.

The relationships between afterload and the velocity of isotonic contraction (+dL/dt) and relaxation (-dL/dt) are shown in Figures 7 and 8, respectively. Contractile velocities recorded for both K-H solutions were significantly greater than observed for shock plasma but not control plasma. Control and shock plasma were different only at the extremes of the afterload weights. In contrast, the relaxation velocity was significantly reduced over the entire range of afterloads relative to the other treatments when shock plasma was tested. The magnitude of this depression over the entire range of afterloads was not less than 67%, 48%, and 45%, respectively, for K-H1, K-H2, and control plasma. This compares with respective values of 54%, 40%, and 32% observed for contraction velocity. Relaxation appears to be more sensitive to the depressant action of shock plasma.

The latency times recorded over the range of afterloads for each solution are shown in Figure 9. There were no significant differences between any of the corresponding points on the curves for latency or for any of the other time indices. However, the time to onset of contraction was always slowest in the shock plasma. The shortest latencies were observed with the initial Krebs-Henseleit solution. The time to the maximum velocity of contraction (TMVC), time to peak of the contraction (TPC), the time to maximum velocity of relaxation (TMVR), and the duration of the contraction (DUR), shown in Figures 10, 11, 12, and 13, all demonstrate the same relationship as observed for latency. Shock plasma consistently caused longer times while the fastest times

were observed with K-H1. No differences between shock and control plasma and K-H2 were observed for the last three time indices, as shown in Figures 11-13. This may suggest that the depressant may have more effect on latency. The final K-H solution was always tested after the two plasmas and yet the times were consistently faster than with the shock plasma. The very marked difference between K-H1 and K-H2 may be indicative of the progressive deterioration of muscle function with time or may represent irreversible damage produced by the shock plasma. The depressant nature of the shock plasma must be partially reversible or the response times to the K-H2 would not have improved.

Figure 14 has been included to summarize the relationship between the various timed events in the contractile cycle with increasing afterload. The curves represent the same curves for K-H2 found in Figures 9-13. Latency and TMVC, which are indices of the contraction or shortening process become longer with increasing afterload. This is consistent with the expenditure of additional energy to overcome the system resistance, the intrinsic stiffness of the muscle, and the inertia of the afterload weight. The reverse occurs during relaxation as the heavier afterloads tend to break cross-bridges and restore the alignment of the papillary muscle to the preload length.

This study established the depressant properties of fresh shock plasma derived from a canine hemorrhagic shock model similar to that incorporated into the early studies of Lefer and Martin (1970). The system for bioassaying the plasma was unique in several respects. The in vitro model was the young canine papillary muscle. Each muscle was tested with fresh control and shock plasma and referenced to pre and

post recordings in Krebs-Henseleit. A diffuser system which had been designed and incorporated into the system facilitated high oxygen tension in the muscle bath without addition of antifoaming additives to the plasmas. Any effects of pH were minimized by the continuous monitoring and correction of change.

Shock plasma does not affect maximum load but does produce a significant depression of relaxation velocity. However, whole plasma may contain many humoral agents, such as the catecholamines, which have positive inotropic properties and which may have antagonized any activity of the depressant factor on the myocardium. Subsequent studies were performed to isolate and bioassay the endogenous shock factor.

ASSESSMENT OF A MYOCARDIAL DEPRESSANT SUBSTANCE IN CANINE HOMOGENATES

Introduction

Plasma obtained from the shock experiments was subsequently subjected to partial separation by gel chromatography and then bioassayed on isotonically contracting papillary muscles. There was no consistency to the data and no conclusions could be inferred from those limited studies. Great variability was the norm in all experiments and no significant depression occurred in the assay of the individual peaks. Individual canine papillary muscles, in general, respond with some variation even when the recorded parameters are tested in the reference Krebs-Henseleit solution. The original protocol of this study had entailed individually screening each plasma from each respective shock experiment. However, the potential for added variability between samples may have further confused the attempts to localize depression in a chromatographed fraction.

Additional factors were also considered which may have further contributed to the inconclusive findings. First, there was a period of between 90-120 days from collection and storage of the plasma until the first separations and the in vitro assessments. Second, the chromatography methods employed a chromatography column which limited the sample volume which could be applied to the column. The possible compounding effects of time and concentration superimposed on the variability between muscles could not be addressed without modifying the protocol substantially.

Failure to observe marked and consistent depression could also have been due to the bioassay methods. Lefer et al. (1967) have employed

isometric preparations of feline muscle with a stimulus frequency of 60/minute and a temperature of 37 C. Canine muscles contracting isotonically at a frequency of 12/minute and at 27 C have been employed in our laboratory. Both methods enabled determinations of maximum isometric tension, hence the type of contraction was initially not deemed of significance. The canine has been used extensively by Lefer et al. as a source for MDF. Therefore, the canine bioassay model should be responsive to the depressant and the canine model was deemed appropriate. Conditions of temperature and frequency of stimulation had been previously assumed to have little influence on the response to the depressant. Brutsaert et al. (1970) and Mattiazzi and Nilsson (1976) have demonstrated that both conditions do have significant effects upon general muscle function. The effects of temperature and frequency of stimulation are two of the many physical factors affecting myocardial contraction which have been extensively reviewed by Blinks and Koch-Weser (1963) and Koch-Weser and Blinks (1963).

Williams et al. (1969) have observed depression of peak developed isometric tension and contractile velocity of rat trabeculae at 37 C. Their results are consistent with observations by Lefer (1970) and Lefer and Rovetto (1970) for feline papillary muscles. However, no depression was observed by Williams et al. when the experiments were conducted at 30 C. Therefore, the temperature of the muscle preparation was an important consideration in the evaluation of the depressant action of shock material. The experimental protocol was altered in lieu of the aforementioned concerns, the inconclusive findings, and the observations reported by Williams et al.

Ferguson et. al (1972) have previously employed pancreatic homogenates subjected to hypoxic conditions for isolation of MDF. They have observed comparable in vitro depression of papillary muscles in response to partially isolated fractions of either shock plasma or pancreatic homogenates. Improved standardization had the potential advantage of decreasing the variability observed with shock plasma. Therefore, experiments were conducted to generate depressant material from homogenated canine pancreas and then partially isolate the depressant fraction by gel chromatography. The effects of temperature and frequency on the response of papillary muscles to the depressant were then assessed in a subsequent study.

Methods for homogenation and processing of the canine pancreas

The pancreas was removed in separate experiments from four large dogs weighing 30.1 $\pm$ 5.4 kg. Each dog appeared healthy prior to anesthetization with pentobarbital (25 mg/kg IV). A midline incision was made and the pancreas was carefully exposed to avoid unnecessary trauma. The pancreas was rapidly dissected free from the attached structures. It was placed in a cold saline solution to bathe the tissue and facilitate washing of blood and debris from the surface during transport. Any superficial connective tissue and fat was dissected from the surface and it was then washed with a modified Krebs-Henseleit solution equivalent in composition to that used for bioassay. Each pancreas was then blotted dry, weighed to the nearest 0.1 g, minced, mixed with fresh Krebs-Henseleit solution (1:6 w/v), and homogenized in a Waring blender for two minutes. This entire process took less than seven minutes. The pancreatic homogenate was quickly divided into two

fractions deemed "Control" and "Shock." Volumes of the respective solutions were determined.

Control homogenate was placed in a water bath at 80 C for 15 minutes to denature the proteins. Debris was removed by centrifuging for 30 minutes at 16,000 x G at 4 C. Clear supernatant was removed, sealed in a polyvinyl jar, and frozen at -10 C. Shock homogenate was placed in sealed jars and incubated in a shaker bath at 37 C for 4 hours. Thereafter, the Shock homogenate was processed like the Control.

Samples of the supernatants were analyzed for osmolarity and calcium, sodium, and potassium concentrations. Osmolarity was generally about 10 mOsm/kg greater than determined for the Krebs-Henseleit solution. Shock homogenate was usually slightly higher than the Control. Sodium concentration in both homogenates was consistently lower than in the Krebs-Henseleit solution and measured between 125-130 meq/l. Potassium concentration was in excess of 15 meq/l. This would reflect the cellular damage and release of potassium caused by homogenization. Calcium concentration was reduced to approximately 4.5 mg/100 ml in the Control and 4 mg/100 ml in the Shock homogenate versus approximately 9.2 mg/100 ml for Krebs-Henseleit. It was concluded that these alterations in composition precluded any attempt to bioassay whole homogenate material.

Respective homogenate material from each dog was thawed, mixed, and centrifuged to remove any debris. Equal volumes of each solution from each dog were then pooled and lyophilized to dryness overnight. The ratio of dried homogenate to volume of supernatant was 0.013 g/ml for Control and 0.022 g/ml for the shock material. Samples were sealed in a

desiccator with desiccant and under a vacuum at -10 C until further processed and bioassayed.

Fractionation of the homogenates

The first step in fractionation was reconstitution of 0.436 g of dried material in 8 ml of distilled water. This value was derived from the average ratio of wet pancreatic weight/kg of body weight divided by the estimated blood volume/kg of body weight. The quantity of depressant substance reconstituted roughly extrapolated to 20 ml (e.g., 20 ml x 0.013 g/ml) of blood which was to be collected in 10 ml fractions when chromatographed and assayed in a 10 ml chamber. Individual peaks were generally localized in 1-3 tubes. This would theoretically be equivalent to assaying approximately a 1-2 fold concentration of plasma, assuming the same quantity of homogenate derived depressant was present per unit of blood as during hemorrhagic shock.

Reconstitued material was thoroughly dissolved and then ultrafiltered at 4 C through an Amicon 50 ml stirred cell mounted with a Amicon UM-2 membrane. This membrane has a 1000 molecular weight cutoff. Nitrogen at 55 psi was used to pressurize the cell. Usually, all but 0.5-1.0 ml of fluid and filtrand was recovered. All the ultrafiltrate was layered on a glass barreled Econo-column with a 2.5 cm I.D. and 75 cm length (Bio-Rad Laboratories). The column was packed with BioGel P-2, 200-400 mesh and maintained at 4 C in a cold room. This packing enabled separation by molecular weight over the molecular weight range of 100-1800 Daltons and has been used in the previous studies by Lefer and Martin (1969). Flow through the columm was maintained constant at

20 ml/hour by a Harvard Apparatus peristaltic pump. Flow rate was checked for 24 hours before and after a separation to verify the flow rate. Ten milliliter fractions were collected with an ISCO circular fraction collector set to cycle every thirty minutes. All glass columms and tubes for collection were siliconized (Siliclaid) to minimize peptide binding. A modified Krebs-Henseleit solution which was equivalent in composition to that used for bioassay less the glucose was used for eluting the samples. Fractionation required 24 hours and was timed to permit bioassay at about 22 hours after layering the column. Specific procedures for preparation of the column, maintenance, and calibration are described in the following section.

Preparation of the chromatography peaks for bioassay

The optical density of each fraction collected was measured at 230 nm using matched quartz cuvettes and either a Beckman Model 21 or a Beckman DB-GT spectrophotometer. An elution chromatogram was then constructed and the peaks to be assayed selected. Small aliquots of each peak were taken for measurement of osmolarity by freezing point depression with an Advanced Osmometer. The calcium concentration was measured by the method described by Annino and Giese (1976). The calcium concentration was adjusted, if necessary, to that of Krebs-Henseleit with microliter quantities of a stock calcium chloride solution. Total change in volume of the chamber fluid did not exceed 1% change in volume per 10% change in the calcium concentration.

All samples were warmed to appropriate temperature, pregassed with 95% O_2, 5% CO_2 and adjusted to pH 7.4 with 0.1 N HCl or 0.1 N NaOH. Aliquots of the solutions were saved upon completion of the bioassay for

subsequent determinations of sodium and potassium concentrations or reevaluation of the osmolarity or calcium concentration.

Preparation of the chromatography columns

Standard glass Econo-Column systems (2.5 cm I.D., 75 cm length, and 368 ml nominal column volume)(Bio-Rad Laboratories) were siliconized prior to packing with Bio-Gel P-2, 200-400 mesh (Bio-Rad Laboratories). All polypropylene adaptors, funnels, caps and other acessories plus the polyethylene tubing were also siliconized.

Dry Biogel P-2 was added to a deaerated modified Krebs-Henseleit solution without glucose which had been previously deaerated. The gel was then allowed to hydrate for four hours at room temperature. Most of the supernatant was removed and replaced with fresh Krebs-Henseleit solution and the gel was gently mixed again. This suspension was then deaerated at a low negative pressure for thirty minutes prior to pouring the column. The hydrated gel suspension was slowly and carefully added to a reservoir mounted on the column which had been previously filled with deaerated Krebs-Henseleit. The stopcock regulating column outflow was opened when approximately five cm of bed had formed. All subsequent operations were completed in a cold room at 4 C.

The height of the column effluent tubing was adjusted to yield a zero hydrostatic pressure head. Column flow was then maintained at 20 ml/hr by a Harvard Apparatus peristaltic pump connected in-line between the column and a four liter reservoir filled with deaerated modified Krebs-Henseleit solution without glucose. Flow was maintained at least 24 hours or until the packed bed height was constant. Excess gel and the reservoir were removed and the column flow maintained and

periodically measured over an additional 48 hours. The excess BioGel was retained and periodically used to replace the top 3-5 cm of the column packing.

Layering procedures

All columns were allowed to flow a minimum of 24 hours prior to application of a sample. This permitted accurate measurement of the flow rate and elution of any residual contaminants. The tubing from the peristaltic pump was disconnected and the outflow tubing clamped over the fraction collector just prior to layering. Buffer was removed from the column down to the packing with a syringe fitted with a long piece of polyethylene tubing. The sample was slowly applied with another syringe to the side of the column and allowed to flow down at a rate which did not disturb the packing. When all the sample had been applied the column was connected to a second peristaltic pump which had been previously calibrated at the same rate but which pumped only air. The column was closely watched until the sample had just been absorbed into the packing. Small volumes of K-H were added to wash residual sample from the walls of the column. Approximately five ml of additional K-H were added after the washing had been absorbed and the column again connected to the system with the K-H reservoir for the duration of the elution.

Maintenance of the column

Columns were periodically cleaned with a 0.1% sodium dodectyl sulfate (SDS) solution. Approximately 10 ml were applied to the column, allowed to enter the packing, and then eluted with K-H for a minimum of 72 hours (1.5 liters). The top 3-4 cm of column packing was replaced

when any discoloration or altered flow rate was noted. When the columns were not to be used for an extended period they were first cleaned and the flow rate was determined over several days. The pump was then stopped, the outflow closed, and the inflow opened to eliminate any pressure. Flow rate was determined when a column was in use again and never varied from the shutdown rate.

Samples of the buffer on top of the column packing, the reservoir fluid, and the column effluent were periodically cultured for bacterial growth but were negative in all cases.

Calibration of the column

The void volumes of the columns were determined periodically by layering each column with bovine serum albumin (BSA). Concentrations and sample volumes ranged from 0.5 ml of a 1% BSA + 3 ml of K-H to 1 ml of %5 BSA + 3 ml of K-H. BSA was detected by measuring aliquots of each collected sample at an optical density of 230 nm with a Beckman Model 21 Spectrophotometer.

Molecular weight calibration was accomplished by applying the following compounds to the columns.

1. Sulfamethazine (MW 278)(Nutritional Biochemicals Corporation): Sulfamethazine was prepared by adding the compound in excess to normal saline, thoroughly mixing, and allowing dissolution overnight in the cold room. The columns were layered with 3.0 ml of the saturated solution.

2. Bacitracin (MW 1411)(Nutritional Biochemicals Corporation): Bacitracin was prepared in saline at a concentration of 1 mg/ml. Columns were layered with 3.0 ml of the solution.

3. Phenol red (MW 376)(Metheson, Coleman and Bell): One ml fractions of a saline solution containing 6 mg/ml of phenol red were applied.

4. Angiotensin I (MW 1296.7)(Sigma Chemical Co.): Three ml fractions of a saline solution containing 5 mg/ml were applied.

5. Oxytocin (MW 1001)(Anpro Pharmaceutical Co., Inc.): Three ml of the oxytocin solution at a concentration of 20 units/ ml were applied to the columns.

All the molecular weight markers except phenol red were detected in the effluent samples by measuring the optical density at 230 nm. Phenol red was measured at 540 nm.

Location of the salt peak has been of prime concern in prior studies. Therefore, the elution volume of the salt peak was detected by layering the column with three ml of a 1 M NaCl solution and then measuring the osmolarity of each sample collected. Location of the salt peak was routinely confirmed each time a test sample was chromatographed when the osmolarity of the peaks was determined.

Methods for the Isometric Screen of Chromatographed Peaks

Nine papillary muscles were prepared by the methods previously described for the bioassay of plasma and utilized to assess the effects of the respective peaks separated by gel chromatography. However, the bioassay system was modified to permit stimulating and recording isometric contractions. Isometric preparations were employed to reduce the prolonged time required for completing afterload curves, to reduce the extensive time required for analysis of data, and hopefully to enable testing of all peaks on the same muscle.

The bioassay system was the same as previously described except for the following modifications. An L-shaped muscle clamp was fabricated from a small block of Plexiglas mounted on the end of a large bore stainless steel tube. The plastic block was sectioned and two 21 gauge stainless steel tubes were embedded in the half of block secured to the large stainless steel tube. Holes were bored in the other half of the block which enabled the two halves to be pinched together securing the wall end of the papillary muscle. The opposite end of the large tubing was securely clamped to the hydraulic cylinder of a David Kopf Instruments, Inc. microdrive. Muscle length could be varied in micron units by raising or lowering the clamp. The upper chordae tendineae end of the papillary muscle was mounted with the same snare device described in the previous study and attached to a Grass model FT.03 force displacement transducer for recording isometric tension. Muscles were stimulated by one electrode placed parallel to the muscle and attached to the main stainless steel tube which supported the clamp and by a second electrode embedded in the surface of the clamp in intimate contact with the muscle.

Solutions were gassed with 95% oxygen and 5% carbon dioxide which was bubbled through two 27 gauge stainless steel tubes inserted into the chamber bottom. Recirculation of fluid was accomplished by connecting the external fluid circuit to two additional 18 gauge tubes inserted into the chamber, such that fluid was returned to the bottom of the chamber and was removed from the top. This enhanced mixing of the chamber fluid. Oxygen tensions were monitored periodically and were in excess of 500 mm Hg.

Each papillary muscle was equilibrated in modified Krebs-Henseleit for no less than one hour. All experiments were conducted at 37 C and a stimulus frequency of 60 per minute. The muscle length was altered by extending or retracting the microdrive unit to which the muscle clamp was secured. Muscle length was initially increased in small increments until the developed tension began to decrease. Resting tension and developed or active tension were then recorded at each step as the length was decreased. These data were then fitted (least squares method) to Equations #1 and #2 with a computer in order to determine the length where maximum tension was developed (Lmax). Each muscle was then adjusted to 90% of Lmax for the duration of the experiment. Muscle length and cross-sectional area were determined by the methods previously described.

Testing of solutions

The respective test solutions (either Krebs-Henseleit or the homogenate peak eluted in Krebs-Henseleit + glucose) were warmed to 37 C, pregassed and adjusted to pH 7.4 prior to bioassay. Approximately 4 or 5 peaks or portions of a peak were tested on each muscle. Every attempt was made to randomize the sequence for testing the respective peaks. Each solution was added to the chamber and the responses and pH monitored until a stable recording was obtained over a ten minute period and then the parameters were measured. Stabilization following each change of solutions was almost always rapid and was complete within 5 minutes. The effects of time over the duration of the experiment were examined by comparing the respective K-H solutions paired with each peak. Effects of time on each comparison were negated by always

comparing the response to each peak to the subsequent K-H. This tended to underestimate any depression which occurred. Several exchanges of K-H were made following each peak to insure removal of any residual material.

Recordings of stimulus artifact and isometric tension were made on a Beckman R611 polygraph. The auxiliary output of the isometric tension channel was differentiated and the change in tension per change in time (dT/dt) was also recorded. The transducer, which was linear up to 10 grams (highest weight tested), was calibrated with known weights before each experiment and the calibration verified at the conclusion. Measurements made from the recordings are illustrated in Figure 3 and are defined as follows.

1. Resting tension (RT) = tension on the muscle due to changes in muscle length.
2. Peak developed tension or active tension (AT) = maximum tension developed in excess of resting tension following stimulation and contraction of the muscle.
3. Velocity of (contraction) developed tension (+dT/dt) = maximum rate of developed tension during contraction phase. Calculated from the slope of the polygraph tracing.
4. Velocity of (relaxation) decline in developed tension (-dT/dt) Calculated from the maximum negative slope on the polygraph tracing.

Results of the Bioassay of the Individual Chromatographed Peaks

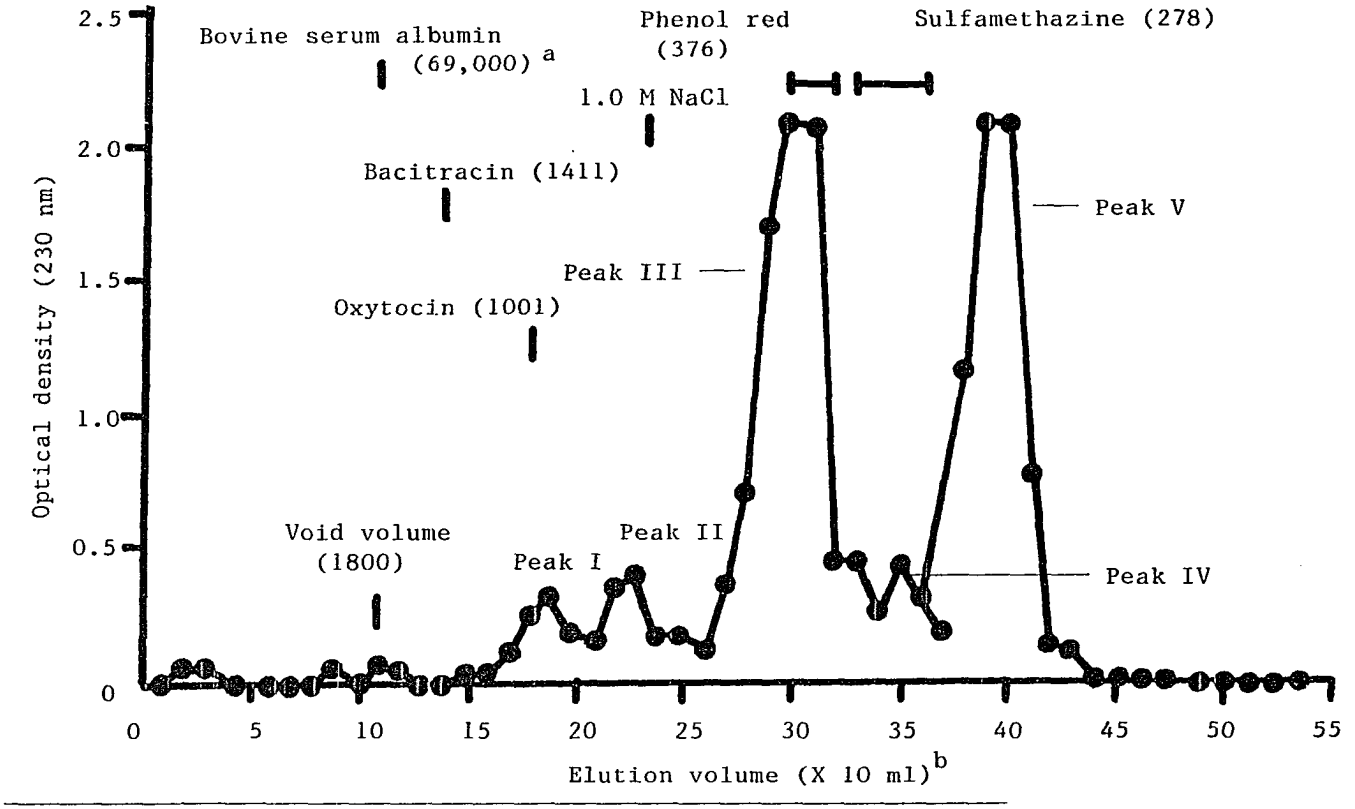
A typical elution profile of a sample of shock pancreatic homogenate together with the elution volumes of the various molecular

weight markers is shown in Figure 15. The elution volumes and molecular weights of the markers were fitted to an exponential regression equation and the molecular weights of the following chromatographed peaks were then extrapolated from the equation.

Peak	Molecular weight
I	962 (range: 850-1000)
II	732
III	376-407
IV	276
V	187-218

The elution volume of bovine serum albumin (BSA) was approximately 110 ml which corresponded to a molecular weight of 1796. Therefore, BSA eluted at a volume that closely matched the upper exclusion limit (1800) for Biogel P-2.

The characteristics of the muscles used to test the chromatographed peaks and the composition of the test solutions have been summarized in Table 5. The muscles had a mean length of 5.49 $\pm$ 1.59 mm (mean $\pm$ S.D. for all subsequent values) and a mean cross-sectional area of 1.39 $\pm$ 0.50 mm^2. No significant differences were noted between muscles used to bioassay each peak. Likewise, there were no differences between the composition of the respective peaks. A majority of the samples which had been saved for electrolyte analysis subsequent to completion of this phase of the project were lost when successive attempts by our technical personnel to analyze them were unsuccessful due to equipment malfunction. Values obtained at a later date did not correlate at all with the anticipated results. This probably reflects contamination of



[a] Molecular weight of calibration markers.

[b] Column packed with BioGel P-2, 200-400 mesh; 0.435 g reconstituted in distilled water and ultrafiltered (mol. wt. < 1000); filtrate layered on column and eluted with Krebs-Henseleit solution (without glucose).

Figure 15. Typical elution profile of a pancreatic homogenate separated by BioGel P-2 chromatography

Table 5. Characteristics of the muscles and composition of the respective chromatographed fractions

PARAMETER	Material Analyzed	Peak or corresponding K-H [a] I	II	III	IV	V
Length (mm)		5.67[b] ± 2.40	5.65 ± 2.17	5.94 ± 2.06	6.25 ± 2.34	4.65 ± 0.53
Cross-sectional area (mm^2)		1.24 ± 0.51	1.57 ± 0.57	1.56 ± 0.52	1.45 ± 0.57	1.51 ± 0.59
Calcium (meq/l)	Peak	2.18[c] ± 0.20	2.28[d] ± 0.05	2.25[e] ± 0.01	2.28[d] ± 0.13	2.30[d] ± 0.25
	K-H	2.33[c] ± 0.05	2.28[d] ± 0.03	2.25[e] ± 0.08	2.28[d] ± 0.03	2.30[d] ± 0.05
Sodium (meq/l)	Peak	143.3[d] ± 1.5	149.3[e] ± 15.4	137.2[e] ± 4.4	140.5[e] ± 4.4	140.0[d] ± 7.0
	K-H	141.0 ± 4.9 (for all peaks)				
Potassium (meq/l)	Peak	5.2[d] ± 0.2	5.9[e] ± 1.4	5.1[e] ± 0.2	5.2[e] ± 0.3	5.1[d] ± 0.1
	K-H	5.0 ± 0.1 (for all peaks)				
Osmolarity (mOsm/kg)	Peak	275.3 ± 2.9	281.0 ± 12.6	280.9 ± 6.5	277.3 ± 8.8	277.8 ± 5.2
	K-H	274.2 ± 6.0	273.8 ± 6.9	275.5 ± 4.5	272.2 ± 4.7	273.3 ± 6.7
Muscle (n)		6	6	8	6	4

[a] Paired t-test for significance; no significant differences.
[b] All values expressed as mean ± S.D.
[c] Number of experiments = n-1.
[d] Number of experiments = n-2.
[e] Number of experiments = n-3.

the samples or changes in composition due to repeated thawing and freezing or evaporation. Although limited samples were analyzed, the sodium and potassium concentration and the osmolarity tended to be higher in Peak II. This would be consistent with the elution of the salt peak at that same elution volume. The calcium concentation of Peak I was approximately 6% less than the corresponding K-H solution and was more variable ($\pm$ 0.8 mg/100 ml) than the other peaks. A decrease in calcium concentration of 1% has been observed to decrease maximum isotonic load (Po) and peak developed isometric tension (active tension) about 1% (unreported data). A small effect of decreased calcium concentration, although not substantial, must be considered in subsequent examination of the responses to Peak I.

Results of the bioassay of each of the respective chromatographed peaks and the corresponding comparisons to the K-H solution are summarized in Table 6. There were no significant differences in muscle function between the respective groups of muscles when tested in K-H solution. The absence of notable differences in muscle characteristics between groups used to test the peaks, the composition of the test solutions, and the muscle function for each group assessed in K-H solution is interpreted as presumptive evidence that any effect of the respective peaks on muscle function was due to a myocardial effect or some component in the peak itself.

When the responses of the muscles to the respective peaks, shown in Table 6, were compared to the responses in the corresponding K-H solution, only Peak I produced any significant depressant activity. Developed tension was significantly depressed by 30.4%, +dT/dt was

Table 6. Results of the bioassay of respective chromatography fractions

PARAMETER[a]	Material Analyzed	Peak or corresponding K-H I	II	III	IV	V
Resting tension (RT) (g/mm^2)	Peak	0.97[b] ± 0.37	0.84 ± 0.52	0.63 ± 0.29	0.60 ± 0.35	0.83 ± 0.39
	K-H	0.99 ± 0.44	0.82 ± 0.46	0.65 ± 0.34	0.61 ± 0.33	0.80 ± 0.36
Developed (active) tension (AT) (g/mm^2)	Peak	0.59[c] ± 0.55	0.59 ± 0.56	0.63 ± 0.39	0.52 ± 0.34	0.39 ± 0.50
	K-H	0.90 ± 0.86	0.60 ± 0.56	0.59 ± 0.39	0.48 ± 0.29	0.69 ± 0.54
Max. rate tension developed (+dT/dt) ($g/sec/mm^2$)	Peak	4.56[c,d] ± 3.42	5.82 ± 3.73	7.73[d] ± 3.14	6.35 ± 2.99	6.42 ± 3.50
	K-H	5.92 ± 3.64	5.72 ± 3.64	6.54 ± 3.17	6.15 ± 2.65	6.38 ± 3.36
Max. rate decline in tension (-dT/dt) ($g/sec/mm^2$)	Peak	6.66[d] ± 9.10	7.24 ± 6.48	8.70[d] ± 4.50	8.31 ± 6.25	8.38 ± 6.62
	K-H	8.86 ± 9.14	7.78 ± 7.52	7.38 ± 4.03	7.63 ± 5.09	6.82 ± 4.83
Latency (LAT) (msec)	Peak	79.6[d] ± 11.3	88.0[c,d] ± 10.7	76.7[d] ± 9.4	80.3 ± 5.2	76.3 ± 10.1
	K-H	78.6 ± 5.2	79.6 ± 10.1	76.4 ± 11.3	84.3 ± 10.7	70.8 ± 9.4

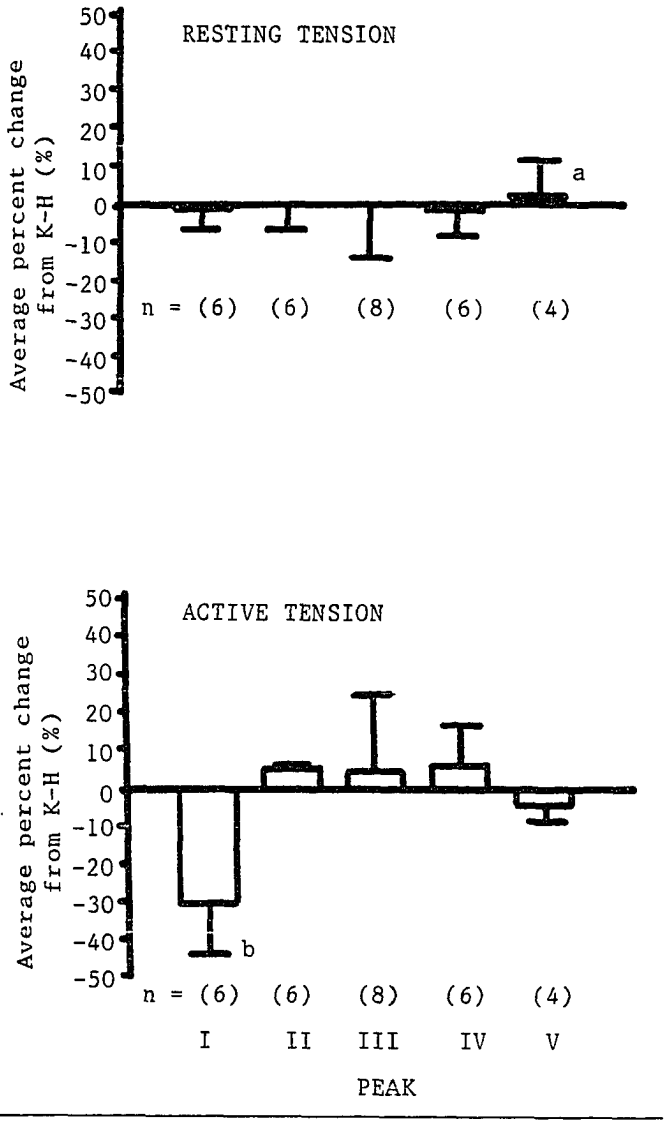
Time to max. +dT/dt (msec)	Peak	156.2[d] ± 61.1	149.3[d] ± 30.0	147.9[d] ± 29.3	133.7 ± 8.8	156.0 ± 45.5
	K-H	151.2 ± 36.3	140.4 ± 10.3	143.0 ± 31.1	135.7 ± 4.2	151.0 ± 35.2
Time to peak tension (TPT) (msec)	Peak	206.4[d] ± 40.0	210.0[d] ± 19.3	224.3[d] ± 23.5	213.3[d] ± 20.4	219.0 ± 29.9
	K-H	214.6 ± 24.1	156.2 ± 104.9	214.1 ± 24.9	213.3 ± 13.2	219.0 ± 29.1
Time to max. -dT/dt (msec)	Peak	278.8[d]	250.9[d]	274.7[d]	25.7 [d]	280.8
	K-H	272.8 ± 55.3	267.8 ± 26.9	265.4 ± 50.4	262.4 ± 10.6	272.5 ± 59.7
Duration (DUR) (msec)	Peak	418.2[d] ± 42.2	418.3[d] ± 30.3	442.1[d] ± 42.9	419.7 ± 34.7	424.3 ± 22.6
	K-H	423.6 ± 35.1	429.8 ± 37.3	439.0 ± 23.6	425.3 ± 38.3	405.5 ± 27.6
Muscles (n)		6	6	8	6	4

[a] Number (n) for each parameter equals the number of muscles.
[b] All values expressed as mean ± S.D.
[c] Significantly different from corresponding K-H; ($p<0.05$); paired t-test for significance.
[d] Number of experiments = n - 1.

decreased by 25.4%, and -dT/dt declined by 34.5 %. The magnitude of depression would have been even greater if data from one muscle with which Peak I had been tested had not been included in the statistical comparison. For example, Peak I depressed developed tension in that muscle by only 8.8% compared to a range of 20 to 52.9 % for the other five muscles. Whether the muscle was not responsive to the depressant or if there was a loss of activity in the peak could not be determined. Therefore, all muscles tested were incorporated into the statistical analysis.

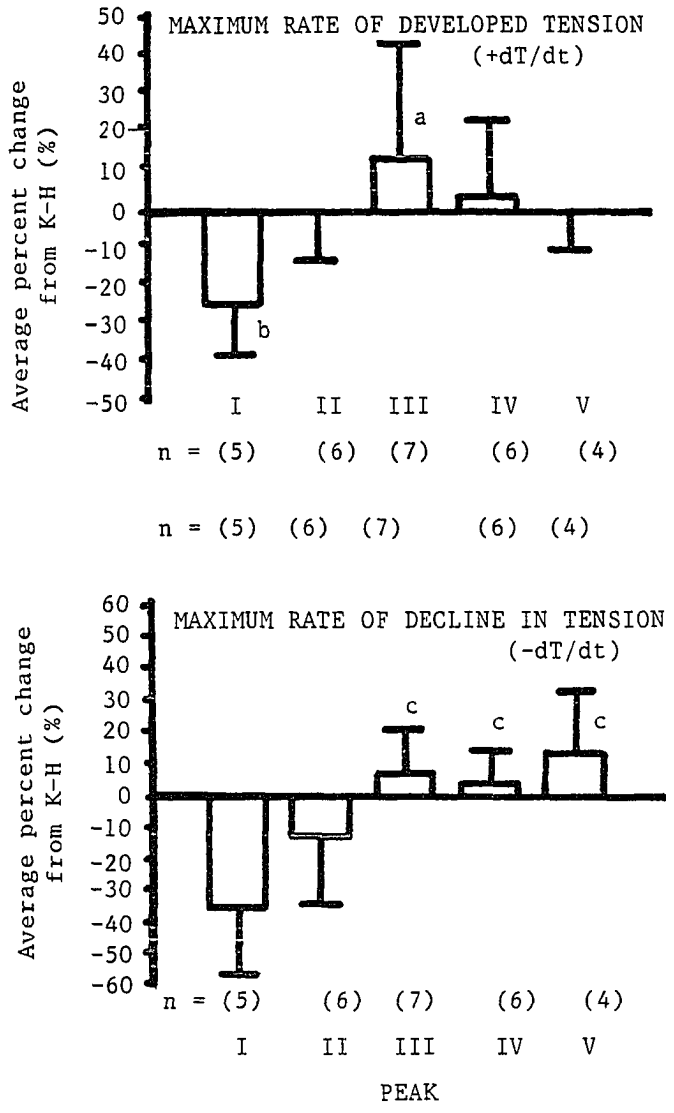
A significantly longer time to onset of contraction (latency) but shorter time to the maximum -dT/dt was observed when Peak II was tested. Although the changes were significant, it should be noted that Peak II did not produce a noticeable change from the previous solutions tested in two experiments. Therefore, one or two additional peaks were tested before K-H was added to the muscle chamber. The comparison between K-H and Peak II included some change which may not have been produced by the peak. Latency and time to peak tension (TPT) were shorter for Peak V. There was less than a 5% difference for both parameters. Since only four muscles were tested and some error in measuring latency times is inherent, any effect of Peak V is questionable. Neither Peak II nor V produced an effect which could be interpreted as myocardial depressant in nature.

The relative magnitude of depression produced by the respective peaks was determined by expressing the data for each peak as a percent change from the corresponding K-H solution. The average percent changes for each respective peak are presented in Figures 16, 17, and 18. The



[a] All values expressed as mean ± S.D.
[b] Significantly different from all other peaks; ($p<0.05$).

Figure 16. Relative change in tension expressed as percent change from corresponding Krebs-Henseleit solution



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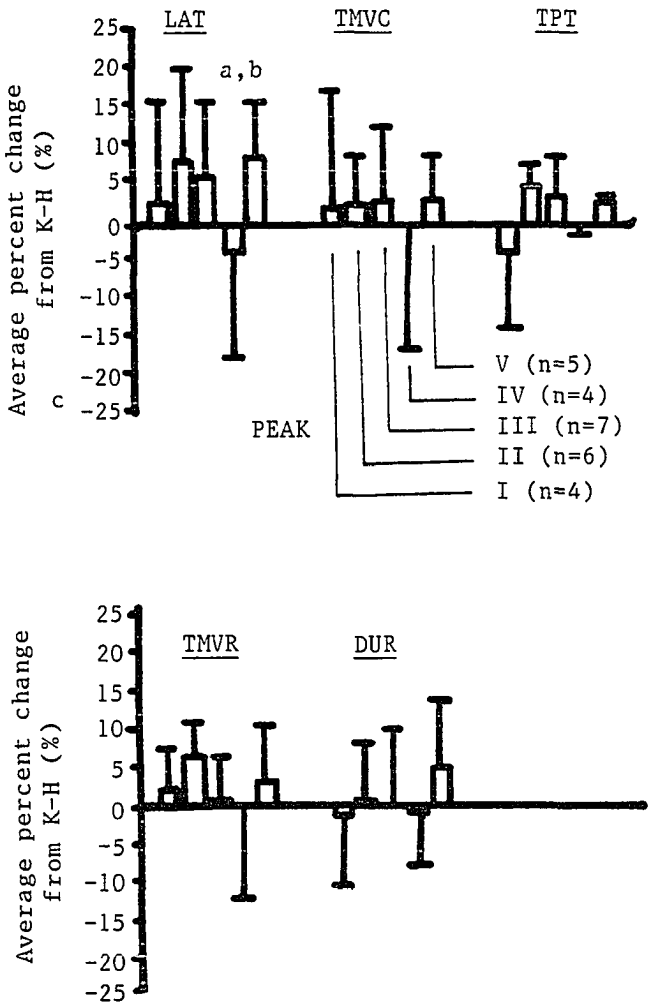
[a] All values expressed as mean ± S.D.
[b] Significantly different from the other peaks; ($p<0.05$).
[c] Significantly different from Peak I; ($p<0.05$)

Figure 17. Relative change in the rate of tension expressed as percent change from corresponding Krebs-Henseleit solution



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[a] All values expressed as mean ± S.D.
[b] No significanct differences.
[c] A negative change indicates a shorter time from stimulus to measured parameters.

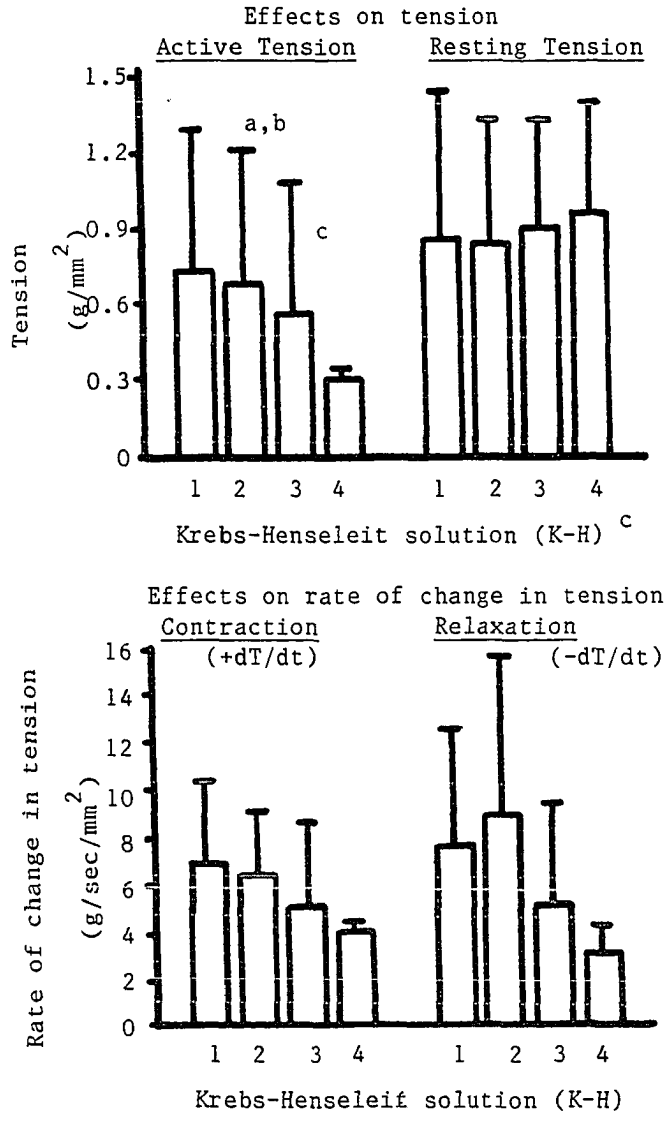
Figure 18. Relative change in latency times expressed as percent change from corresponding Krebs-Henseleit solution

active tension in response to Peak I decreased despite no marked decrease in the resting tension. Considerable variation was noted in the relative effect of Peak III, although the mean response was a small increase in resting tension. These changes may have been caused by the corresponding shift in the resting tension. This pattern of net but variable increase in active tension with Peak III was of interest because it is consistent with countless observations made during preliminary studies with shock material obtained by multiple methods and from several sources. Bioassay efforts were previously concentrated on that peak for several reasons. First, there was similarity to the elution profile to that reported by A.M. Lefer (personal communication during a visit to our laboratory). Second, the early isolation of the samples eluted at that elution volume exhibited mild depressant activity. Finally, the molecular weight calculation had been in error and the error detected only after calibration and separation was performed on columns of different dimension (i.e., the columns used for the present study).

Figure 17 illustrates the relative change in both +dT/dt and -dT/dt for the respective peaks. Depression was only observed with Peak I. A net increase in both parameters was observed for Peaks III, IV, and V. Peak II, which caused an increase in active tension, caused a 10% depression of the +dT/dt but only a small net depression of the -dT/dt. Peak II had a higher osmolarity and potassium concentration than the other peaks. However, osmolarity of approximately 375 mOsm/kg and potassium concentration of approximately 9 meq/l did not produce any changes in active tension, +dT/dt, or -dT/dt in preliminary studies.

Relative changes in the latency times are shown in Figure 18. Despite Peak I effects on active tension and dT/dt, there was no consistent effect on the latency time intervals. No significant differences or pattern were noted. A small and nonsignificant decrease in the time to the peak of the isometric contraction was the only change in which Peak I appeared to differ in any way from the others. It is also possible that no changes would be anticipated because less tension (fewer cross-bridges) is developed. Formation of fewer cross-bridges (less tension) would require a shorter period to achieve maximum cross bridge formation. However, the decrease in +dT/dt caused by Peak I would imply that the rate of formation and turnover of cross-bridges would also be slower. Alternately, the failure to distinguish a significant effect on one of the latency times may reflect imprecision in measurement. At least two individual measurements of different contractions were made and the results averaged. While the measurements were usually quite close (less than 3% difference), the substantial variability, which may be noted in Figure 18, may be the result of the inherent error in measuring distance on a small chart even with a measuring device reported accurate to $\pm$ 0.025 mm.

The effects of time over the 4-8 hour span of a typical experiment were evaluated by comparing the individual muscle responses to the respective K-H solutions tested following each peak. Results of these comparisons are presented in Figures 19 and 20. Although there was a tendency for active tension, +dT/dt, and -dT/dt to decline; there were no significant differences between any of the four K-H solutions for any these parameters. It should be noted that the relative decline in



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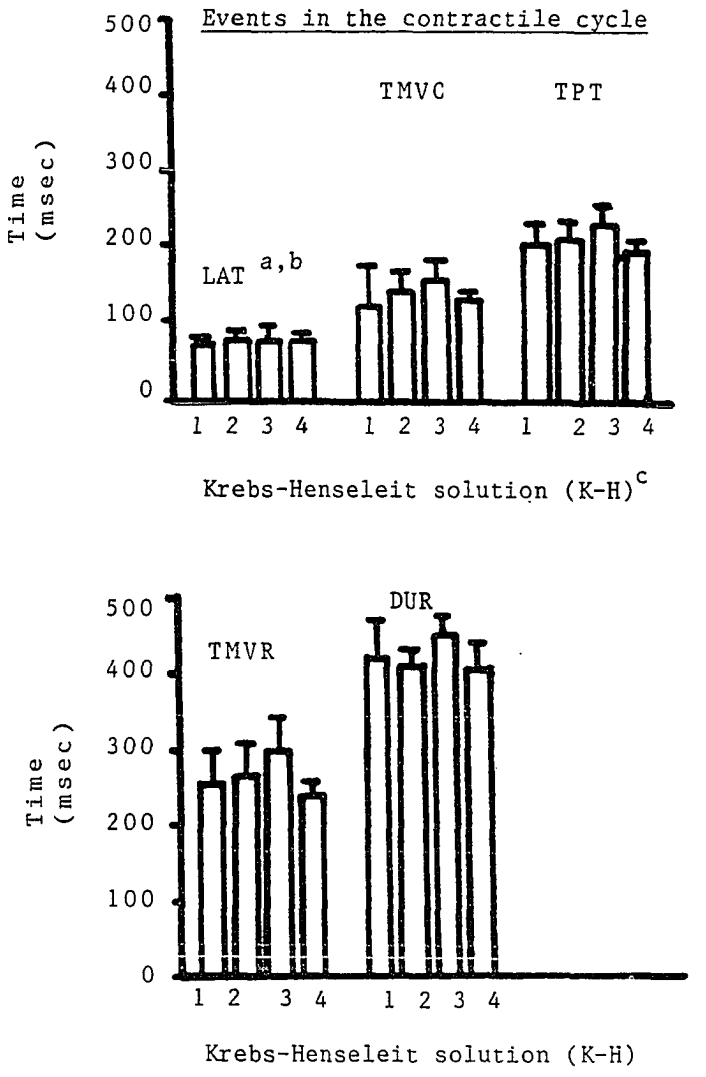
[a] All values expressed as mean ± S.D.

[b] No significant differences; analysis of variance for significance.

[c] Number of experiments: n = 9 for K-H1 and K-H2, n = 5 for K-H3, and n = 3 for K-H4.

Figure 19. Changes in tension over the duration of the experiments



[a] All values expressed as mean ± S.D.

[b] No significance differences; analysis of variance for significance.

[c] Number of experiments: n = 9 for K-H1 and K-H2, n = 5 for K-H3, and n = 3 for K-H4.

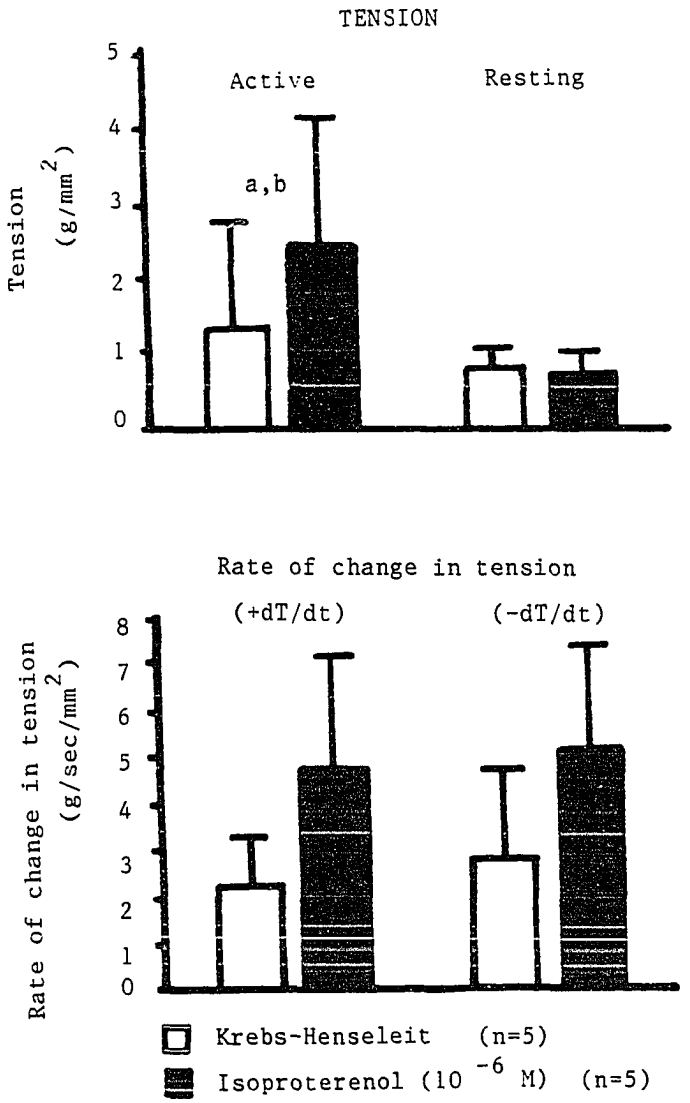
Figure 20. Changes in time to events in the contractile cycle over the duration of the experiments

-dT/dt with K-H3 and K-H4 appears to be of a greater magnitude than observed with +dT/dt. A consistent observation, in this and prior studies, has been the greater sensitivity of relaxation to either the depressant substance or the cumulative effects of time. All of the various latency times, shown in Figure 20, are consistent and do not seem to correlate with the changes in active tension or the generation and decline of tension.

The ratio of active tension to resting tension was calculated for each muscle and K-H solution. These ratios were then statistically compared and no significant differences were present. When only the measurements for muscles in which all four K-H solutions were tested, the ratio ranged from 0.43 $\pm$ 0.18 for K-H1 to 0.37 $\pm$ 0.14 for K-H4.

Deterioration of muscle function over time would not appear to be a factor in assessing the depressant properties of the respective peaks by the methods described. It should be recalled that each peak was referenced to a K-H solution which was subsequently assayed. Therefore, any negative effects of time were negated at the risk of underestimating the magnitude of depression. This means of comparison also eliminates any assessment of the reversibility of the depressant.

The effects of isoproterenol (10^{-6} M) on five of the papillary muscles were assessed after testing the respective peaks. The results are shown in Figures 21 and 22. Isoproterenol had no effect on resting tension but increased active tension by 70%, +dT/dt by 109%, and -dT/dt by 84%. These responses were consistent for all muscles tested. All latency intervals were shortened, although not significantly, by isoproterenol. Such observations are consistent with the anticipated



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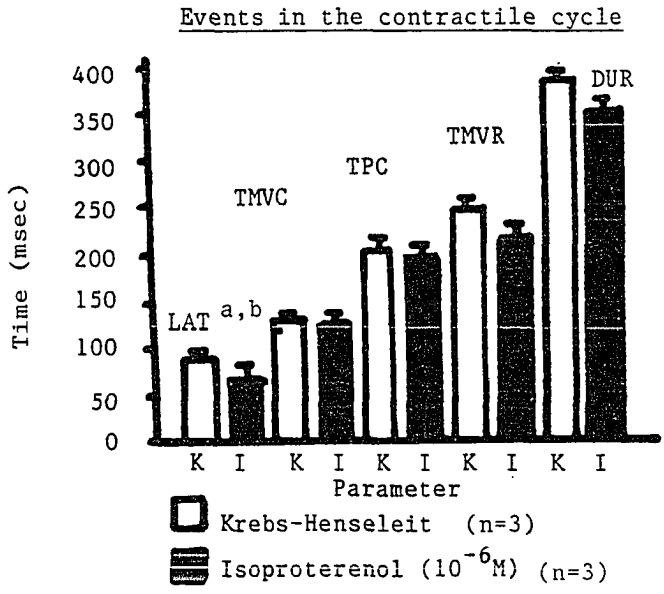
a All values expressed as mean ± S.D.
b Significant difference; ($p<0.05$).

Figure 21. Effects of isoproterenol (10^{-6} M) on tension



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[a] All values expressed as mean ± S.D.
[b] No significant differences; t-test for significance.

Figure 22. Effects of isoproterenol (10^{-6} M) on the various latency times

effects on myocardial tissue both *in vivo* and *in vitro*. The response to isoproterenol was transient with the peak response lasting no more than about one minute. Most of the muscles began to deteriorate rapidly following isoproterenol administration, despite repeated exchanges with fresh K-H solution. This irreversible damage may reflect the limitations on oxygen diffusion which is inherent in isolated muscle preparations. The increased metabolism induced by isoproterenol could exacerbate any limitation of diffusion and result in a hypoxic muscle. All the tested papillary muscles responded to the standard dose of isoproterenol even after 4-8 hours of experimentation.

Conclusions

Pancreatic homogenates subjected to shock-like conditions were separated into five fractions by gel chromatography. Only Peak I, with a molecular weight between 500-1000, had a depressant effect on the peak developed or active tension and both the rate developed tension, and relaxation of tension of isometrically contracting canine papillary muscles at conditions of 37 C and a stimulus frequency of 60 per minute. The molecular weight of Peak I is consistent with the range reported by Lefer and Martin (1969) for MDF. Location of the depressant in Peak I in the present study contrasted to the fourth peak (Peak D) for MDF. The difference in elution profiles, despite very similar methods which included the same ultrafiltration process and chromatography packing, cannot be explained. Possible explanations could include the differences in processing he homogenates, denaturation, differences in storage conditions, and the use of a pump to drive the separation.

Peak I was the only fraction to significantly depress the active

tension developed by the canine papillary muscles. Lefer et al. (1967) quantitated the depressant action of MDF as depressant units, each equivalent to a 1% decrease in peak developed or active tension. Therefore, even the magnitude of depression in the present study (30%) compares with the 30-50% range for MDF. A lower concentration of calcium in the peak sample, although not significantly different from K-H, could not account for more than 10% of the observed depression. The depressant effect of Peak I on both contraction and relaxation velocity is also consistent with the observations in the initial assessment of shock plasma.

This study confirms the presence of a depressant substance derived from a pancreatic homogenate which is either similar to or possibly MDF. Partial isolates of the pancreatic homogenate have not previously been reported to depress the contractile function of young canine papillary muscles, despite the frequent use of the canine as a source for such depressant material. Relaxation veloity has also been shown to be a reliable parameter for assessing the depressant property. Data obtained from subsequent studies relative to relaxation may provide important clues as to the mechanism of action of the substance. This phase of the project established the presence and location of the depressant in the chromatogram which facilitated a subsequent study of the interaction of the bioassay conditions of temperature and frequency of stimulation.

EFFECTS OF TEMPERATURE ON THE ISOMETRIC CONTRACTION OF CANINE PAPILLARY MUSCLES

Introduction

Langendorff (1895) and Snyder (1908) were two of the first researchers to report the inotropic effects of temperature on isolated cardiac muscle. A most comprehensive review of physical factors which affect muscle function, such as temperature and frequency of stimulation, has been written by Blinks and Koch-Weser (1963). Cooling has been shown by, Clark (1920), Hajdu and Szent-Gyorgyi (1952), Saunders and Sanyal (1958), Kelly and Hoffman (1960), Monroe et al. (1962), MacLeod and Koch-Weser (1963), Parmley and Sonnenblick (1969), Mattiazzi and Nilsson (1976), and Frist et al. (1978) to increase the strength of contraction and the duration of the contraction cycle over a temperature range of approximately 15-37 C. The time for cardiac muscle to reach peak tension also is prolonged, as reported by Reiter (1972). Kababgi and Schneider (1980) have not observed temperature, over a range of 22-38 C, to have any effect on resting muscle length or tension of unstimulated rat ventricular strips. They did observe increased resting tension with cooling down to 28 C when the muscles were electrically stimulated. Kruta (1938a, 1938b) has reported that the rate of shortening or isotonic contractile velocity is decreased as the temperature is reduced. Brutsaert et al. (1970) confirmed a significant inhibition of maximum velocity of isotonic contraction by feline papillary muscles produced by cooling them from 37 C to 29 C. Maximum load was inversely related to temperature, although their results were not significant. A similar effect on the force-velocity curve was

reported for rabbit papillary muscle by Edman et al. (1974). Edman et al. suggested that temperature alters both the degree of activation of the contractile elements and the rate of interaction of the cross bridges. Cooling also has a more pronounced effect on prolonging the relaxation phase of a muscle contraction than on the development of tension, as shown by Berne (1954), Hirvonen (1955), Hirvonen and Lybeck (1956), and Goldberg (1958). Finally, prolongation of the duration of the myocardial action potential has been reported by Hollander and Webb (1955), Cranefield and Hoffman (1958), and Kelly and Hoffman (1960). The effects of cooling on the action potential are more marked at lower stimulus frequencies and may be overcome by increasing the frequency. A review of the literature confirmed that temperature has a marked effect on cardiac muscle function both in vitro and in vivo.

The shock homogenate derived depressant in the previous group of experiments was evaluated using an isometric preparation and protocol similar to Lefer et al. (1967). Subsequent experiments were planned to evaluate the interaction of temperature and frequency on the negative inotopic effect on canine myocardium using an isotonic preparation. However, a series of preliminary experiments were conducted to confirm the effects of temperature on the isometric contraction of the canine muscles utilizing the bioassay system previously described. The responses of the isometric preparations could then be used as a means of comparison to the isotonic observations in the studies to follow.

Methods: Examination of the Effects of Temperature

Papillary muscles from fourteen young canine weighing approximately 1.5-3 kg were prepared for recording isometric contractions by the

methods described in the previous section. All experiments were conducted using only the modified Krebs-Henseleit solution, previously described, to bathe the muscles. Muscles were equilibrated initially at either 27 or 37 C and a stimulus frequency of 12 per minute until stable contractions were recorded over a 30 minute period. Equilibration time at 27 C required at least one hour more than at 37 C. Muscle lengths were subjectively adjusted towards Lmax until the muscles had equilibrated. A preload curve was then constructed and used to set the muscle length to 90% Lmax. All subsequent experimentation was conducted at that length established at the initial temperature.

Temperature of the system was adjusted by resetting the thermostat which controlled the water bath temperature. The water was either heated or the water in the circulating bath was exchanged for cooler water and then heated to 27 C before being circulated. It required less than three minutes after recirculating the water through the system for the bioassay system to reach the new temperature. All muscles were equilibrated a minimum of 30 minutes after the system was adjusted regardless of the temperature and for at least 15 minutes of stable recordings when the initial period was not sufficient. The initial temperature was alternated between 27 and 37 C in successive experiments.

Fresh Krebs-Henseleit solution which had been pregassed at the appropriate temperature was added to the chamber to bathe the muscle at the beginning of each test. The response at each temperature was recorded on the polygraph after stable recordings had been observed for 20 minutes, the temperature was readjusted, and the procedure repeated.

All measurements for tension and velocity were normalized for cross-sectional area. Data were statistically analyzed by a paired t-test for significance.

Results and Discussion

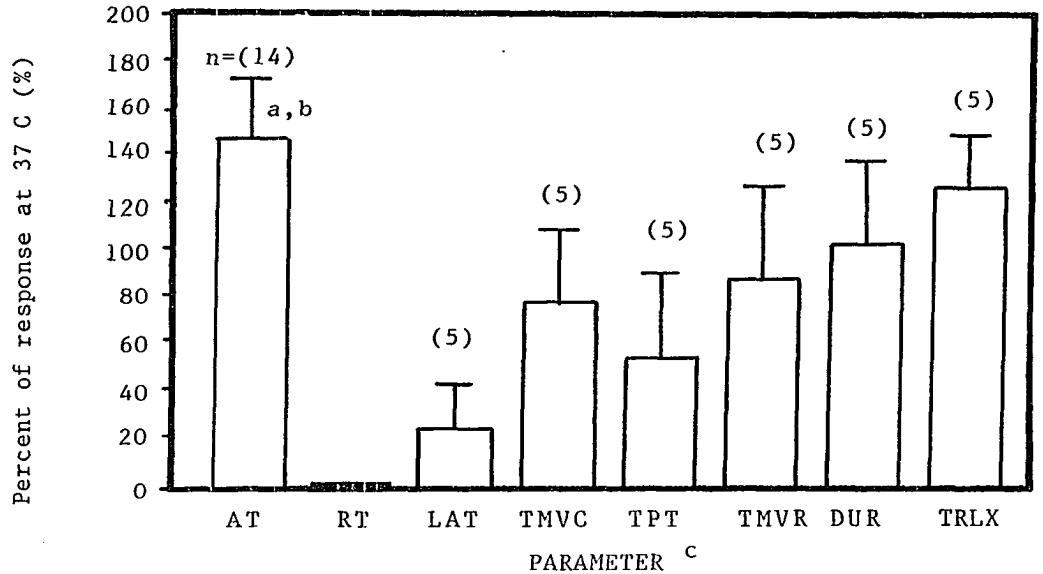
The results of the experiments have been summarized in Table 7. These results clearly confirm the positive inotropic effect of cooling on cardiac tissue. Relative effects of cooling on muscle function, expressed as percent change from the parameter mean at 37 C, are summarized in Figure 23. Active tension was doubled by the decrease in temperature. In contrast, the resting tension was essentially unchanged. All of the measured time intervals to respective states in the contraction cycle were significantly prolonged by cooling. Insufficient data were collected for statistical evaluation of velocity. Although active tension and the time indices were all significantly altered by cooling, it may be observed in Figure 23 that the largest quantitative changes were in active tension, latency, duration of contraction, and the time from peak tension to completion of the contraction. Lewartowski et al. (1974) reported no difference in the Lmax of feline papillary muscles at 23 and 30 C and only a small decrease in resting tension with cooling. The absence of an effect on resting tension is in contrast to the observations of Kababgi and Schneider (1980) for rat ventricular strips.

The increased active tension observed with cooling has been attributed to an increased intracellular concentration of free calcium. According to Mattiazzi and Nilsson (1976), free intracellular calcium is the net result of the rate of calcium release, the duration of release,

Table 7. Comparison of the effects of temperature on the isometric contraction of canine papillary muscles

PARAMETER	N	27 C	37 C
Active tension (AT) (g/mm^2)	(n=14)	2.22[a,b] ± 1.60	1.11 ± 0.93
Resting tension (RT) (g/mm^2)	(n=14)	0.98 ± 0.48	0.99 ± 0.55
Latency (LAT) (msec)	(n=5)	70.2[b] ± 31.4	34.6 ± 24.9
Time to maximum +dT/dt (msec)	(n=5)	184.4[b] ± 20.4	124.6 ± 35.8
Time to peak tension (TPT) (msec)	(n=5)	375.0[b] ± 59.0	209.8 ± 25.6
Time to maximum -dT/dt (msec)	(n=5)	555.6[b] ± 146.9	292.6 ± 45.6
Time for relaxation (TRLX) (msec)	(n=5)	481.8[b] ± 159.1	208.2 ± 51.5
Duration (DUR) (msec)	(n=5)	856.8[b] ± 211.5	418.0 ± 59.9
TPT/TRLX	(n=5)	0.82[b] ± 0.19	1.03 ± 0.22
+dT/dt ($g/sec/mm^2$)	(n=3)	8.08 ± 4.13	9.22 ± 7.17
-dT/dt ($g/sec/mm^2$)	(n=3)	5.89 ± 3.24	10.06 ± 9.40
Cross-sectional area (XSA) (mm^2)	(n=14)	1.19 ± 0.40	

[a] All values expressed as mean ± S.D.
[b] Significant temperature effect; ($p<0.05$).



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[a] All values expressed as mean ± S.D.
[b] No statistics applied.
[c] Parameters are defined as follows: AT = developed tension; RT = resting tension; LAT = latency, TMVC, as used here, refers to maximum +dT/dt; TPT = time to peak tension; TMVR, as used here, refers to maximum -dT/dt; DUR = duration of contraction; and TRLX = time for relaxation (or DUR-TPT).

Figure 23. Relative effects of cooling to 27 C on the isometric contraction of canine papillary muscles

and the rate of resequestration by the sarcoplasmic reticulum. It is the amount of free calcium which interacts with the contractile elements to produce tension. The longer latency time at 27 C would imply that it took longer for sufficient sarcolemmal depolarization and a sufficient transcellular flux of calcium to trigger release of the intracellular stores of calcium. Although the duration of the action potential was not measured in these experiments, it would be consistent with the prolongation of the contraction cycle. Therefore, the critical sarcoplasmal calcium concentration would be maintained for a longer contraction. Finally, it has been shown by Harigaya and Schwartz (1969) that cooling induces an inhibition of the reuptake of calcium by tne sarcoplasmic reticulum. This inhibition and those of other enzymatic dependent processes of contraction and relaxation are thought to be additional mechanisms for maintenance of calcium levels which results in both increased cross-bridge formation and the duration of interaction. Relaxation can only occur when the sarcoplasmic calcium concentration falls. The ratio between the time to develop and time to dissipate tension (time to peak shortening/time to relaxation) was calculated for each temperature and is given in Table 7. The relaxation process was longer at lower temperature and approximately equal to the contraction time at 37 C. Relaxation appears to be more sensitive than contraction to the influence of cooling. Langer and Brady (1968) proposed that the inhibition of relaxation could also be due to some undefined mechanism which overloads the ability of the relaxing system to remove the increased sarcoplasmic calcium concentration associated with muscle cooling. Relaxation of feline papillary muscles has been reported by

Frist et al. (1978) to be more resistant to the effects of hypoxia at 29 C than at physiological temperature.

It can be concluded from these experiments that canine papillary muscles are significantly affected by temperature. The response to cooling is both qualitatively and quantitatively similar to values reported for muscle from other species. The data obtained under isometric conditions provided a basis for comparing the effects of temperature on isotonic contraction to be examined in subsequent experiments. Temperature does have significant effects on muscle function. The potential effects of temperature on the response to the depressant material were examined in the subsequent study.

INTERACTION OF TEMPERATURE AND FREQUENCY ON THE RESPONSE TO A DEPRESSANT FACTOR

Introduction

A myocardial depressant substance was identified in the previous studies which tended to confirm the findings of Lefer (1970), Lefer (1974), Lefer and Spath (1977), Williams et al. (1969), Fisher et al. (1973), Okuda and Fukui (1974), Rogel and Hilewitz (1978), Goldfarb et al. (1979), and Okada et al. (1983). Whole shock plasma from dogs subjected to hemorrhagic shock, but not control plasma or a modified Krebs-Henseleit solution, was observed to depress maximum isotonic load and both contraction and relaxation velocity. The depressant activity was partially isolated by gel chromatography in a molecular weight fraction corresponding to a molecular weight between 500-1000. This fraction or chromatography peak was subsequently shown to depress the peak isometric tension and the rate of tension onset and decline of isometrically contracting canine papillary muscles when the stimulus frequency was 60 per minute and at a preparation temperature of 37 C. These conditions were incorporated into the protocol of Lefer et al. (1967) for the assessment of the activity of myocardial depressant factor (MDF).

The primary purpose of this study was to determine if either temperature or frequency of stimulation of the isolated cardiac muscle preparation could be factors which influence whether the depressant substance produces a negative inotropic effect. Depression has been reported in the literature primarily when conditions are more physiological (i.e., 37 C and a stimulus frequency of 60 per minute).

The inotropic effects of temperature and frequency may mask the effect of the shock induced depressant substance at conditions of 27 C and a frequency of 12 per minute. Williams et al. (1969) evaluated shock material on rat ventricular tissue and observed depression of peak isometric tension and velocity at 37 C and at a stimulus frequency of 60 per minute. However, when the temperature was decreased to 30 C, no depressant effect was observed. The effect of temperature was not further evaluated. Absence of an effect at the lower temperature and frequency would confirm the observations by Urschel et al. (1972). They were unable to detect a depressant effect of shock derived material at the lower temperature and frequency. It would also provide an explanation for the relative absence of any consistent depression observed in almost two years of frustrated efforts in our laboratory using many methods to identify a depressant of pancreatic origin in the canine, feline, bovine, porcine, and ovine. Finally, it would help to resolve the controversy which still exists concerning the existence of an endogenous myocardial depressant substance. Although there are other possible explanations for the discrepancies, temperature and stimulus frequency have been shown to alter muscle function and affect the response of isolated cardiac tissue to various inotropic agents.

Physiologists and pharmacologists have traditionally studied cardiac tissue in vitro at temperatures less than physiological and at low frequency because of the greater stability of the preparation. However, the response to many pharmacological agents may not be uniform at all temperatures. Cotten and Cooper (1962) and Saunders and Sanyal (1958) have reported that the positive inotropic effects of the cardiac

glycosides are decreased by cooling. Similar observations have been reported by Booker (1960), Brown et al. (1962), Martinez and McNeil (1977), and Duncan and Broadley (1978) for the catecholamines. Webb (1950) observed that the negative inotropic effects of acetylcholine on rabbit auricle are increased by cooling. Blinks and Koch-Weser (1963) suggested in their review that the positive inotropic effect of cooling could theoretically increase the muscle contraction sufficiently that a pharmacological agent may not be able to cause any further increase in function. Cooling would be expected to alter the stiffness of the cellular components, decrease the transmembrane fluxes of ions, and inhibit the metabolic processes within the cell. Inhibition of both active and passive processes would be expected to increase the duration of the contractile cycle but also sustain a high intracellular calcium concentration. This elevation of calcium is considered the mechanism for the positive inotropic effect of cooling. Velocity would be lowered and tension increased by cooling due to an increased sarcoplasmic calcium concentration. Harigaya and Schwartz (1969) have reported an inhibition of calcium uptake by preparations of cardiac sarcoplasmic reticulum as temperature is decreased. Conversely, at physiological temperature there would be no inhibition of enzymatic activity and the sarcoplasmic calcium concentration would be quickly lowered by the sarcoplasmic reticulum. Therefore, a negative inotropic effect should be more apparent. Frist et al. (1978) used this rationale to explain a more pronounced effect of hypoxia at physiological temperature. They hypothesized that a higher sarcoplasmic calcium is maintained at low temperature which inhibits reuptake. The temperature effect

predominates and masks the inhibition of reuptake due to hypoxic inhibition of the metabolic mechanisms in the sarcoplasmic reticulum.

Frequency has been reported by Abbott and Mommaerts (1959) and Hill (1951) to increase the degree of activation of cardiac muscle, but Abbott and Mommaerts (1959) have also shown that the duration of the active state is also shortened. Longhurst and McNeil (1980) have confirmed these early observations. Koch-Weser (1963) has reported that the duration of the muscle action potential is inversely related to frequency. The decrease in the duration of the contraction with increased frequency is thought to decrease the entry of calcium per contraction, but the net calcium influx per time is increased. As frequency is increased beyond some critical rate, the sarcoplasmic reticulum might not be able to reduce the sarcoplasmic calcium completely and a positive inotropic effect would be observed. Abbott and Mommaerts (1959) clearly demonstrated that increasing the frequency of stimulation shifted the force-velocity curve away from the origin. Brutsaert et al. (1970) also observed a significant increase in both maximum load and velocity when the stimulus frequency applied to feline papillary muscle was increased from 12 per minute to 24-30 per minute. Sonnenblick (1962a, 1962b) earlier reported that the velocity increased relatively more than the strength or force of contraction. Relaxation velocity of guinea pig papillary muscles was shown by Kapel'ko et al. (1982) to be increased more than contraction velocity when the stimulus frequency was increased from 30 to 120 per minute.

The interaction of temperature and frequency may produce a variable response by cardiac tissue. For example, Cranefield and Hoffman (1958)

have reported that increased frequency opposes the longer duration of both the action potential and the duration of the contraction produced by cooling. Further, Kruta (1938a), MacLeod and Koch-Weser (1963), and Koch-Weser and Blinks (1963) all reported that when either temperature or frequency is held constant, there is an optimum for the other variable at which contraction is maximal.

Frequency would be expected to affect the response of isolated tissue to inotropic agents. Sanyal and Saunders (1958) and Moran (1962) have shown that many pharmacological agents, such as the glycosides, have a more rapid onset of action at higher frequency. Drugs like the catecholamines, strophanthin, acetylcholine, alpha adrenergic antagonists, and metabolic inhibitors may produce positive, no effect, or even a negative inotropic effect at different frequencies, as reported by Lee (1954), Vane (1957), Furchgott and Sleator (1954), Katzung et al. (1957), Koch-Weser and Blinks (1962), and Benfey (1979). The potential for an interaction of temperature and frequency on the expression of any negative inotropic effect on the isolated canine myocardium is well supported in the literature. In general, most published reports of depression produced by the various endogenously produced myocardial depressant factors, like MDF, have been conducted at 37 C and a frequency of 60 per minute. Negative findings, in contrast, were associated with lower temperatures. The possible interaction of temperature or frequency on the response of the canine papillary muscles was evaluated in the following experiments.

Methods

Source of the depressant material

Pancreatic homogenate material was drawn from the same pools of lyophilized homogenates described in the previous section. The methods for reconstitution and chromatographing the samples were also previously described. The first peak (Peak I) was the only peak which exhibited significant depressant activity. Therefore, testing in this study was restricted to a comparison of that peak to a modified Krebs-Henseleit solution at combinations of high and low temperature and frequency.

Preparation of the samples for assay

Separation of the samples by gel chromatography was timed to permit bioassay approximately 20 hours after the sample had been applied to the chromatography column. The osmolarity and calcium concentrations were determined prior to bioassay. Calcium concentration in the Peak I sample (eluted in modified Krebs-Henseleit less glucose) was adjusted to that of the Krebs-Henseleit solution if the concentration varied by more than 5%. Osmolarity of both Peak I and Krebs-Henseleit and other peaks and tubes adjacent to Peak I were also measured. The osmolarities of Peak I and Krebs-Henseleit were always nearly identical.

The bioassay system was the same one which was previously described for assessment of shock plasma. Samples were pregassed with 95% oxygen and 5% carbon dioxide by connecting the test tubes containing the respective solutions in series with the gas line used to gas the muscle bath. Tubes containing the samples were prewarmed to either 27 or 37 C by placing them in a block heater. Sample pH was always checked with a pH meter and the sample regassed prior to adding the solution to the

muscle bath. The pH could be varied by changing the rate of gas bubbling or by the addition of 0.1N NaOH or HCl. However, the two solutions never varied more than 0.010 pH units. Pregassing and temperature equilibration facilitated a very smooth exchange of solutions in the muscle bath and minimized the equilibration time of the muscle (approximately 30-60 seconds).

Muscle bioassay

Papillary muscles from nineteen young canines (thirteen for the shock derived material, and six for the control) weighing approximately 1.5-3 kg were prepared for recording isotonic contraction by the methods described for the assessment of shock plasma. Muscles were equilibrated at the initial combination of temperature and frequency to be tested until stable contractions were recorded over a 30 minute period. Equilibration time at 27 C required at least one hour more than at 37 C. Muscle length was subjectively adjusted during the equilibration period to reflect a muscle length closer to Lmax. Preload curve data were obtained when the muscle was deemed equilibrated and used to compute 90 % Lmax. All subsequent experimentation was conducted at the muscle length (90% Lmax) established at the initial combination of temperature and frequency. Preload length of feline muscles is not affected by temperature, as reported in Lewartowski et al. (1974). Kababgi and Schneider (1980) have shown that the resting tension of rat myocardium, in contrast, increases as the temperature decreases or frequency increases within the ranges employed in this study.

Chromatographed Peak I samples of either control or shock pancreatic homogenate were tested at temperature and frequency of stimulation

combinations of either 27 or 37 C and 12 or 60 per minute. Each experiment with shock homogenate was intended to test all combinations of temperature and frequency. However, in preliminary studies it was observed that high temperature was detrimental to canine papillary muscles. This may have been due to the large muscle cross-sectional area typical of canine papillary muscles. Oxygen demand would be greater at 37 C but delivery may have been compromised by the relatively large distance for oxygen diffusion. Therefore, the working life of an isolated muscle was reduced and sometimes only 2 or 3 comparisons could be made on a given muscle. The sequence for testing combinations of temperature or frequency was partly randomized. Only one of the two factors was changed in sequence and the sequence for testing two specific treatment combinations was reversed in successive experiments. Specific test sequences were chosen in those experiments where all combinations could not be tested and it was necessary to complete a minimum number of experiments within a treatment group and insure that the sequence for testing each treatment was equal.

Temperature of the system was adjusted by resetting the thermostat which controlled the water bath temperature and exchanging the water in the water bath if the subsequent temperature was to be lower. Actual equilibration of the muscle bath temperature required less than three minutes once the circulating pump had been turned back on. Equilibration time for the muscles after changing either temperature or frequency varied but was generally longer at low temperature and frequency of stimulation. A minimum equilibration period of at least 30 minutes was observed at all treatment combinations.

Procedure for testing

Each muscle was tested only with material obtained from one chromatographic separation. Adjacent tubes which comprised Peak I were pooled and the sample used to test at each respective combination of temperature and frequency was taken from that pool. If the volume was insufficient to enable each test to be performed with fresh solution, then some of the solution which had just been tested was added to the fresh solution to insure equivalent volumes. Several experiments were performed in which the depressant substance was removed, the muscle equilibrated in Krebs-Henseleit, and then the same depressant material was added back to the muscle bath. The responses appeared identical. Therefore, it was assumed that any reuse of small quantities of previously tested material would not adversely affect the results.

Following addition of the test material to the muscle bath and adequate equilibration, the muscles were afterloaded. The first priority was to identify the maximum afterload (Po) which just prevented shortening. Muscles were more stressed with the temperature and frequency changes in these experiments than muscles used in the previous study. Recordings of muscle contracting in response to the heavier afterload weights were made sequentially without completely removing the afterload between individual weights to minimize the stress. If Po was approximately 4 g, then approximately 0.5 g was removed and the response recorded following the recording at 4 g. This would be repeated several times until approximately 2 g remained. The afterload weights were then removed, the muscle was rested for ten contractions, and the next afterload weight tested. This procedure greatly reduced the time to

conduct the afterload measurements and noticeably reduced the incidence of muscle fatigue.

Parameters which were directly recorded included stimulus artifact, isotonic shortening, and the isotonic shortening signal differentiated with time (dL/dt). Frequent measurements of time, temperature, and all muscle afterloads were also recorded. All responses were recorded on a Beckman R611 polygraph. It should be noted that the pH of the circulating chamber was continuously monitored with an Orion Research Model 601A Digital pH meter and never varied.

The response of the muscles to isoproterenol (10^{-6} M) was evaluated at the conclusion of all experiments and referenced to the last Krebs-Henseleit solution tested. Isoproterenol was tested as a standard reference and an indicator of muscle responsiveness following the stresses over time and also due to temperature and frequency changes. Muscle length and weight were measured and these values used to determine muscle cross-sectional area by the methods described for the bioassay of shock plasma.

Analysis of results

The recordings of the isotonic contractions were analyzed by the methods described for the bioassay of shock plasma (Measured parameters and calculations). Each group of data was then fitted to a third order polynomial equation (Equation #6). Normalization of data between muscles was accomplished by calculating from each polynomial equation the respective values for each parameter which corresponded to ten percent increments of Po.

Data were statistically analyzed by the ISU Statistical Consultation

Laboratory and with Daisy Professional by Rainbow Computing, Inc., a statistical software program designed to run on the Apple IIe microcomputer. Statistical comparisons were made with the appropriate application of a paired or unpaired Student t-test or the Mann-Whitney-Wilcoxon paired-signed-ranks test (Natrella (1963)) for nonparametric data.

Results and Discussion

The characteristics of the muscles and the composition of the solutions used for the bioassay of either the depressant peak from the shock or control homogenate are shown in Table 8. There were no significant differences between any of the groups for any parameter except osmolarity. The osmolarity of the control peak and the corresponding Krebs-Henseleit solution were significantly greater than for other groups but not from each other. Raising the osmolarity by only 8 mOsm/kg should not have caused a detectable effect on any of the parameters subsequently measured.

Effects of the control homogenate peak

Examples of the elution profiles for the control and shock pancreatic homogenates separated in these studies are shown in Figure 24. Homogenate samples were reconstituted according to the formula defined previously in the study to assess the presence of a myocardial depressant in the homogenates. Shock homogenate separated into five distinct peaks, as previously reported. Only Peak I exhibited significant depressant activity. Control homogenate rarely had any peaks except at an elution volume of approximately 300-360 ml. All identifiable peaks were bioassayed, but either did not depress the

Table 8. Summary of muscle parameters and solution composition for the assessment of temperature and frequency effects

Parameter	SHOCK HOMOGENATE				CONTROL
	F12,27C [a]	F12,37C	F60,27C	F60,37C	All samples
Muscles (n)	8	6	11	10	6
Length (mm)	6.9 [b] ± 1.6	6.8 ± 1.8	6.6 ± 0.9	7.0 ± 1.3	6.2 ± 0.8
Cross-sectional area (mm^2)	1.51 ± 0.44	1.34 ± 0.26	1.52 ± 0.93	1.43 ± 0.33	1.25 ± 0.33
Calcium (meq/l)					
Peak	2.39 ± 0.21	2.39 ± 0.24	2.29 ± 0.23	2.28 ± 0.25	2.23 ± 0.08
Krebs-Henseleit	2.40 ± 0.22	2.43 ± 0.25	2.41 ± 0.18	2.42 ± 0.18	2.19 ± 0.15
Osmolarity (mOsm/kg)					
Peak	273.8 ± 5.1	272.0 ± 4.3	275.6 ± 4.9	275.2 ± 4.7	283.0[c] ± 4.4
Krebs-Henseleit	275.5 ± 3.5	274.2 ± 2.7	275.5 ± 3.6	275.1 ± 3.3	283.0[c] ± 3.3

[a] F = frequency (12 or 60/minute); C = temperature (27 or 37 C).
[b] All values expressed as mean ± S.D.
[c] Significantly different from all corresponding values for shock homogenate, ($p<0.05$).

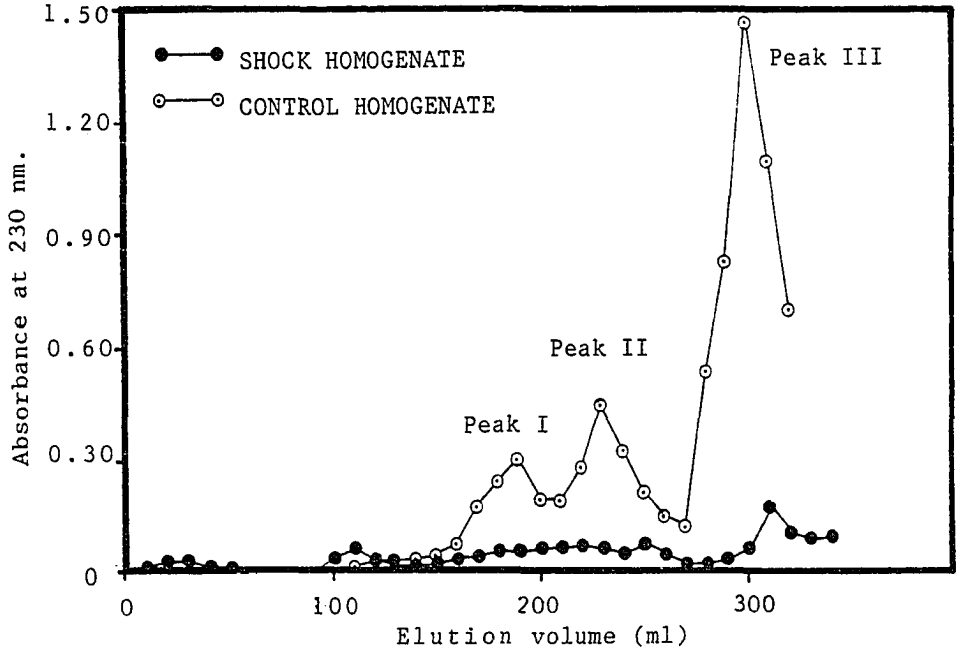


Figure 24. Typical elution profiles of the control and shock pancreatic homogenates separated on BioGel P-2 chromatography columns

papillary muscles or actually caused some improved function. The control elution volume corresponding to Peak II also had a greater osmolarity and contains the salt peak. The profile for control homogenate does not include a detectable Peak I. However, samples with the equivalent volume were bioassayed.

Control pancreatic homogenate which had been processed and separated by the same methods used for the shock pancreatic homogenate was bioassayed on six muscles. The results of these experiments have been summarized in Table 9. Although each experiment was designed to test all four combinations of temperature and frequency, the number successfully completed for each ranged from one to five. Therefore, the data were pooled by either frequency or temperature. For example, experiments at both frequencies conducted at 27 C were pooled and statistically compared to the pooled data at 37 C.

Control pancreatic homogenate exhibited very little effect on any parameter at any combination of temperature and frequency. Improved function was not uncommon. The effects of the control homogenate peak have been expressed as a percent change from the corresponding Krebs-Henseleit solution and are shown in Figures 25 and 26. Maximum load was not significantly affected at either condition of temperature or frequency. If all the data were pooled, then only a 2.45 $\pm$ 10.8% increase (stimulation) of maximum load was observed. Contraction velocity (shortening velocity) also was not affected by frequency. However, contraction velocity increased 4.6 $\pm$ 0.1% when the pancreatic homogenate fraction was tested at 37 C compared to a decrease of 7.7 $\pm$ 9.8% observed at 27 C. Relaxation velocity was not altered when the

Table 9. Summary of the effects of the control homogenate peak, frequency, and temperature on muscle function

	PARAMETER[a]							
	Po (g/mm^2)	Vc ($g/sec/mm^2$)	Vr	LAT	TMVC	TPC (msec)	TMVR	DUR
FREQ = 12/minute (n=6)								
Control Peak	2.68 ± 1.25	2.94[b] ± 1.96	8.07 ± 8.40	70 ± 37	204 ± 82	435 ± 85	571 ± 105	819 ± 145
Krebs-Henseleit	1.87 ± 1.07	3.12 ± 2.15	8.04 ± 8.55	70 ± 33	194 ± 90	429 ± 80	565 ± 111	797 ± 152
FREQ = 60/minute (n=4)								
Control Peak	2.47 ± 0.91	4.85 ± 2.37	8.94 ± 5.14	67 ± 17	151 ± 75	381 ± 121	516 ± 145	692 ± 231
Krebs-Henseleit	2.18 ± 0.70	5.26 ± 2.01	7.65 ± 5.70	72 ± 18	150 ± 57	362 ± 108	507 ± 159	681 ± 233

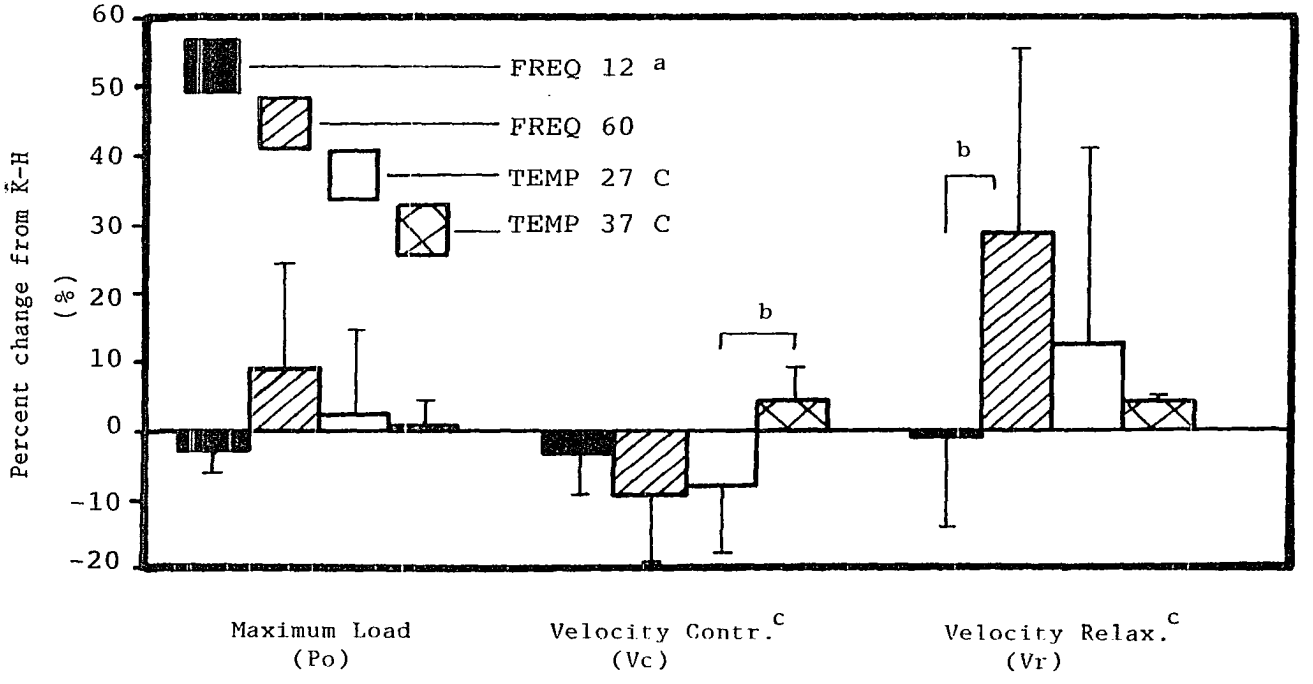
TEMP = 27 C (n=8)

Control Peak	2.68 ± 1.25	3.42 ± 1.59	8.13 ± 6.93	73 ± 34	194 ± 90	463 ± 27	607 ± 36	822 ± 64
Krebs-Henseleit	2.57 ± 1.26	3.81 ± 1.83	7.56 ± 7.27	73 ± 31	178 ± 78	448 ± 38	602 ± 55	857 ± 84

TEMP = 37 C (n=2)

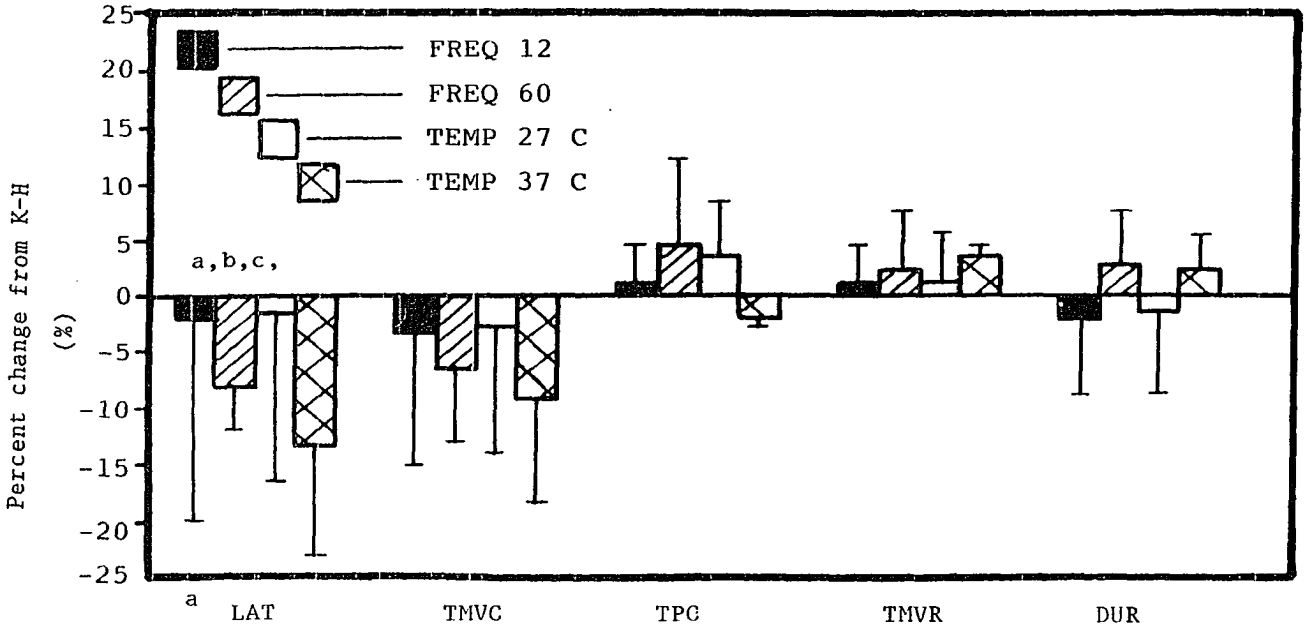
Control Peak	1.20 ± 0.48	4.85 ± 4.90	9.16 ± 9.42	55 ± 12	160 ± 15	257 ± 21	362 ± 19	491 ± 85
Krebs-Henseleit	1.18 ± 0.37	4.63 ± 4.68	9.54 ± 9.75	63 ± 4	152 ± 13	261 ± 26	349 ± 21	480 ± 94

[a] Statistics applied for comparisons where n = 4 or more; no significant differences.
[b] All values expressed as mean ± S.D.



[a] All values expressed as mean ± S.D.
[b] Significantly different; ($p<0.05$).
[c] Velocity of muscles with no afterload.

Figure 25. Effects of frequency and temperature on the relative change in maximum load and velocity in response to the control homogenate peak



a All values expressed as mean ± S.D.
b No significant differences; t-test for significance.
c All times recorded with no afterload on the muscle.

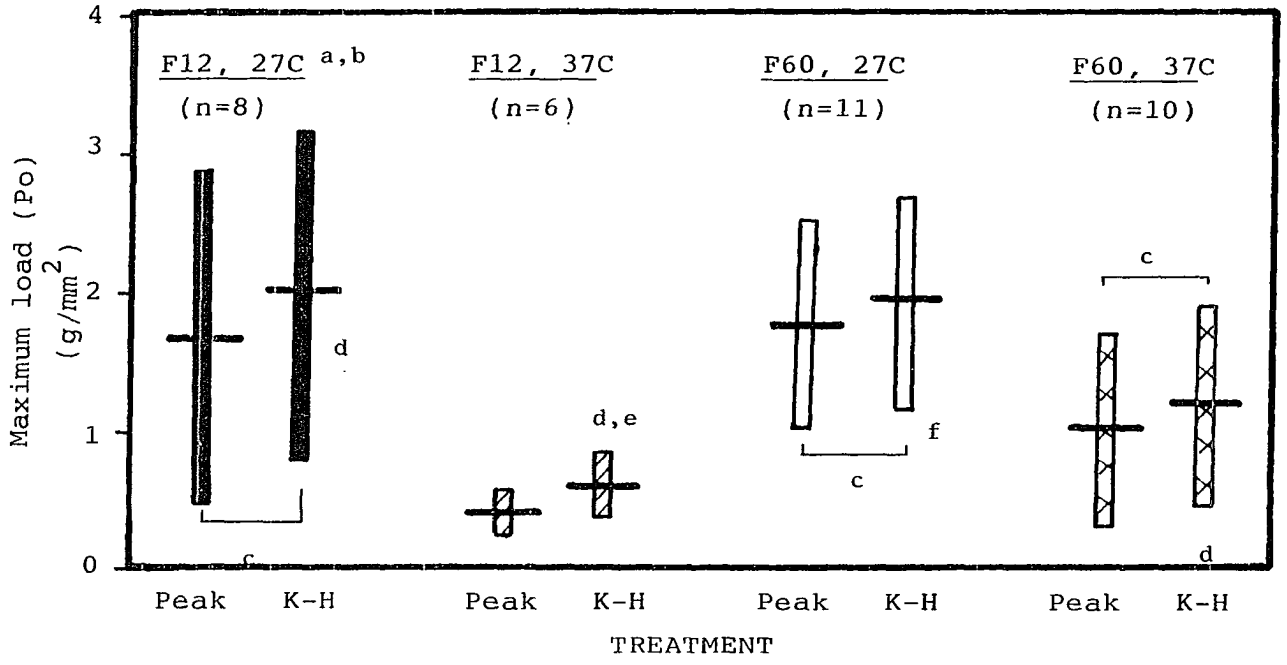
Figure 26. Relative effects of frequency, temperature, and control homogenate peak on latency times

fraction was tested at either temperature but significantly increased from a net depression at low frequency (-0.3 ± 12.59%) to a net activation (28.93 ± 26.47%) at high frequency. Temperature and frequency had no effect on the various latency times of the muscles in response to the pancreatic homogenate, as shown in Figure 26.

Relatively little net change from Krebs-Henseleit solution was observed for the chromatographed fraction of control pancreatic homogenate which corresponded to the shock homogenate fraction evaluated in the isometric screen of the peaks and which was further evaluated in this study. The control homogenate could have and probably should have received a more extensive evaluation. Although the number of experiments was limited by availability of quality muscles and an adequate supply of the original lyophilized control homogenate for subsequent separation, there was a consistent lack of substantial depression. This especially contrasted to the effects on relaxation velocity observed in the subsequent evaluation of the shock homogenate peak. Control pancreatic homogenate did not appear to contain a myocardial depressant substance.

Effects of the shock homogenate peak, frequency, and temperature

The data from the experiments to evaluate frequency and temperature effects on the response to the shock homogenate peak have been summarized in Figure 27 and Tables 10 through 16. All recorded velocity and time data from the individual experiments were normalized for cross-sectional area and fitted to a third order polynomial equation. Values were then extrapolated from the fitted equation at 0.1 increments of maximum load (Po). The coefficients of the equations have not been



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a All values expressed as mean ± S.D.
b F = frequency (12 or 60/minute); C = temperature (27 or 37 C).
c Significant difference between indicated pairs; paired t-test for significance; (p 0.05) significance; ($p<0.05$).
d Significantly different from other means with the same letter; ($p<0.05$).
e Significantly different from K-H at F60, 27C; ($p<0.05$).
f Significantly different from K-H at F60, 37C; ($p<0.05$).

Figure 27. Summary of the effects of shock peak, frequency, and temperature on maximum load (Po)

Table 10. Summary of the effects of shock homogenate, frequency, and temperature, on contraction or shortening velocity

Normalized Relative Load (P/Po)[a]									
.0P	0.1P	0.2P	0.3P	0.4P	0.5P	0.6P	0.7P	0.8P	0.9P
						Note: velocity at 1.0P = 0			
FREQ 12, 27C (n=8)[b]									
					VELOCITY (mm/sec/mm^2)[c]				
Shock Peak									
2.05[d,e] ± 1.34	1.75[e] ± 1.13	1.34[e] ± 0.97	1.29[e] ± 0.85	1.18[e] ± 0.76	1.04 ± 0.68	0.90 ± 0.61	0.73 ± 0.52	0.54 ± 0.40	0.29 ± 0.23
Krebs-Henseleit									
2.48 ± 1.73	2.13 ± 1.45	1.85 ± 1.22	1.61 ± 1.03	1.39 ± 0.88	1.20 ± 0.75	1.01 ± 0.64	0.82 ± 0.52	0.60 ± 0.39	0.35 ± 0.24
FREQ 12, 37C (n=6)									
Shock Peak									
1.81[e] ± 0.72	1.68[e] ± 0.69	1.57[e] ± 0.66	1.49[e] ± 0.64	1.38 ± 0.60	1.26 ± 0.55	1.1 ± 0.48	0.92 ± 0.40	0.68 ± 0.31	0.39 ± 0.20
Krebs-Henseleit									
2.16 ± 0.84	2.05 ± 0.70	1.92 ± 0.96	1.76 ± 0.53	1.58 ± 0.48	1.38 ± 0.44	1.16 ± 0.42	0.93 ± 0.41	0.68 ± 0.40	0.43 ± 0.36

FREQ 60, 27C (n=11)

Shock Peak

3.08	2.42	1.95	1.83	1.58	1.37	1.08	0.95	0.72	0.43
± 2.14	± 1.78	± 1.78	± 1.50	± 1.27	± 1.06	± 0.87	± 0.71	± 0.56	± 0.41

Krebs-Henseleit

3.16	2.68	2.30	1.98	1.72	1.48	1.25	1.01	0.75	0.44
± 2.27	± 1.90	± 1.60	± 1.34	± 1.12	± 0.97	± 0.93	± 0.62	± 0.47	± 0.27

FREQ 60, 37C (n=10)

Shock Peak

2.82[e]	2.54	2.30	2.09	1.89	1.69	1.46	1.18	0.87	0.47
± 1.97	± 1.74	± 1.56	± 1.40	± 1.26	± 1.11	± 0.95	± 0.76	± 0.54	± 0.29

Krebs-Henseleit

3.06	2.69	2.38	2.12	1.87	1.64	1.39	1.13	0.83	0.46
± 1.93	± 1.57	± 1.31	± 1.12	± 0.99	± 0.89	± 0.79	± 0.68	± 0.53	± 0.31

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[a] Calculated as 0.1 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded velocity versus afterload data were fitted to third order polynomial equation and velocities then extrapolated from the equation at 0.1 increments of Po.

[d] All values expressed as mean ± S.D.

[e] Significantly different from Krebs-Henseleit, ($p<0.05$).

Table 11. Summary of the effects of shock homogenate, temperature, and frequency on relaxation velocity

Normalized Relative Load (P/Po)[a]									
.0P	0.1P	0.2P	0.3P	0.4P	0.5P	0.6P	0.7P	0.8P	0.9P
FREQ 12, 27C (n=8)[b]									
				RELAXATION VELOCITY (mm/sec/mm^2)[c]					
Shock Peak									
4.08[d,e] ± 2.69	4.85[e] ± 3.65	5.16[e] ± 4.17	5.09[e] ± 4.24	4.69[e] ± 3.97	4.06[e] ± 3.43	3.27[e] ± 2.73	2.38[e] ± 1.99	1.48[e] ± 1.27	0.62[e] ± 0.59
Krebs-Henseleit									
5.74 ± 3.86	6.69 ± 5.22	7.06 ± 5.90	6.95 ± 5.96	6.41 ± 5.53	5.60 ± 4.80	4.55 ± 3.84	3.37 ± 2.80	2.15 ± 1.77	0.98 ± 0.80
FREQ 12, 37C (n=6)									
Shock Peak									
3.01[e] ± 1.77	2.89[e] ± 1.55	2.75[e] ± 1.36	2.57[e] ± 1.18	2.36[e] ± 1.02	2.10[e] ± 0.87	1.81[e] ± 0.74	1.46[e] ± 0.63	1.06[e] ± 0.50	0.59 ± 0.36
Krebs-Henseleit									
4.92 ± 2.38	4.85 ± 2.18	4.64 ± 2.02	4.29 ± 1.85	3.84 ± 1.66	3.31 ± 1.43	2.72 ± 1.18	2.09 ± 0.92	1.45 ± 0.69	0.81 ± 0.63

FREQ 60, 27C (n=11)

Shock Peak

4.62 ± 2.60	4.80 ± 2.93	4.76 ± 3.04	4.52 ± 2.93	4.12 ± 2.64	3.58 ± 2.22	2.93 ± 1.74	2.22 ± 1.25	1.44 ± 0.84	0.72 ± 0.39

Krebs-Henseleit

5.49 ± 3.35	5.60 ± 3.50	4.46 ± 3.53	5.11 ± 3.35	4.59 ± 2.98	3.94 ± 2.48	3.20 ± 1.92	2.39 ± 1.36	1.56 ± 0.84	0.76 ± 0.38

FREQ 60, 37C (n=10)

Shock Peak

5.80 ± 5.31	5.87 ± 5.59	5.66 ± 5.44	5.23 ± 4.92	4.62 ± 4.16	3.89 ± 3.26	3.07 ± 2.34	2.20 ± 1.51	1.39 ± 0.87	0.62 ± 0.40

Krebs-Henseleit

6.43 ± 3.79	6.36 ± 4.05	6.04 ± 4.15	5.52 ± 3.99	4.85 ± 3.60	4.07 ± 3.03	3.23 ± 2.36	2.37 ± 1.67	1.54 ± 1.04	0.78 ± 0.52

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[a] Calculated as 0.1 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded velocity versus afterload data were fitted to a third order polynomial equation and velocities then extrapolated from the equation at 0.1 increments of Po.

[d] All values expressed as mean ± S.D.

[e] Significantly different from corresponding Krebs-Henseleit, ($p<0.05$).

Table 12. Summary of the effects of shock homogenate, frequency and temperature on latency

	Normalized Relative Load (P/Po)[a]				
	.0Po	.02Po	.04Po	.06Po	.08Po
FREQ 12, 27C (n=8)[b]					
	LATENCY (msec)[c]				
Shock Peak	87[d] ± 20	145[e] ± 13	186[e] ± 24	227 ± 41	283 ± 55
Krebs-Henseleit[f]	84 ± 14	158 ± 16	179 ± 21	219 ± 26	275 ± 29
FREQ 12, 37C (n=6)					
Shock Peak	59 ± 5	85 ± 9	105 ± 10	127 ± 12	153 ± 17
Krebs-Henseleit[g]	66 ± 8	85 ± 7	104 ± 8	124 ± 9	148 ± 17

FREQ 60, 27C (n=11)					
Shock Peak	78 ± 12	124 ± 17	154 ± 20	181 ± 21	222 ± 23
Krebs-Henseleit [h]	75 ± 11	113 ± 16	145 ± 17	176 ± 18	215 ± 21
FREQ 60, 37C (n=10)					
Shock Peak	61 ± 14	80 ± 14	102 ± 8	125 ± 17	149 ± 14
Krebs-Henseleit [i]	57 ± 13	80 ± 15	101 ± 18	122 ± 21	147 ± 23

[a] Calculated as 0.2 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded time versus afterload data were fitted to third order polynomial equations and time then extrapolated from the equation at 0.2 increments of Po.

[d] All values expressed as mean ± S.D.

[e] Significantly different from corresponding Krebs-Henseleit; ($p<0.05$).

[f] Significantly different from g, h, and i at all afterloads; ($p<0.05$).

[g] Significantly different from h and i at all afterloads; ($p<0.05$).

Table 13. Summary of the effects of shock homogenate, frequency and temperature on time to the maximum velocity of contraction (TMVC)

	Normalized Relative Load (P/Po)[a]				
	0	0.2Po	0.4Po	0.6Po	0.8Po
FREQ 12, 27C (n=8)[b]			TMVC (msec)[c]		
Shock Peak	211[d] ± 52	239 ± 63	274 ± 70	315 ± 68	364 ± 63
Krebs-Henseleit	209 ± 51	225 ± 59	275 ± 62	309 ± 51	355 ± 54
FREQ 12, 37C (n=6)					
Shock Peak	177 ± 32	173 ± 28	184 ± 28	205 ± 28	229 ± 25
Krebs-Henseleit[e]	167 ± 28	170 ± 26	181 ± 28	199 ± 29	224 ± 32

FREQ 60, 27C (n=11)					
Shock Peak	198 ± 59	206 ± 56	226 ± 55	256 ± 57	292 ± 56
Krebs-Henseleit[e,f]	187 ± 40	199 ± 53	220 ± 29	248 ± 28	273 ± 21
FREQ 60, 37C (n=10)					
Shock Peak	154 ± 50	155 ± 29	171 ± 52	194 ± 58	214 ± 40
Krebs-Henseleit[e,f]	148 ± 56	159 ± 55	172 ± 53	187 ± 50	204 ± 26

[a] Calculated as 0.2 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded time versus afterload data were fitted to third order polynomial equations and time then extrapolated from the equation at 0.2 increments of Po.

[d] All values expressed as mean ± S.D.

[e] Significantly different from Krebs-Henseleit at FREQ 12, 27C at all afterloads; ($p<0.05$).

[f] Significantly different from Krebs-Henseleit at FREQ 12, 37C at all afterloads; ($p<0.05$).

Table 14. Summary of the effects of shock homogenate, frequency and temperature on time to peak of contraction (TPC)

	Normalized Relative Load (P/Po)[a]				
	0	0.2Po	0.4Po	0.6Po	0.8Po
FREQ 12, 27C (n=8)[b]					
	TPC (msec)[c]				
Shock Peak	439[d] ± 34	445 ± 44	453 ± 51	457 ± 57	455 ± 60
Krebs-Henseleit	434[e] ± 38	435[e] ± 42	440[e] ± 51	444[e] ± 56	440[e] ± 53
FREQ 12, 37C (n=6)					
Shock Peak	242[f] ± 18	237[f] ± 13	235[f] ± 12	234[f] ± 12	238 ± 17
Krebs-Henseleit	245[g] ± 13	240[g] ± 12	236[g] ± 12	234[g] ± 13	235[g] ± 150

FREQ 60, 27C (n=11)					
Shock Peak	349 ± 33	345 ± 30	345 ± 32	345 ± 33	343 ± 32
Krebs-Henseleit	326[h] ± 36	320[h] ± 31	321 ± 27	325 ± 25	325 ± 29
FREQ 60, 37C (n=10)					
Shock Peak	206 ± 33	213 ± 28	219 ± 32	222 ± 34	224 ± 32
Krebs-Henseleit	208[h] ± 30	209[h] ± 31	211 ± 31	212 ± 31	211 ± 32

[a] Calculated as 0.2 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded time versus afterload data were fitted to third order polynomial equations and time then extrapolated from the equation at 0.2 increments of Po.

[d] All values expressed as mean ± S.D.

[e] Significantly different from all other K-H solutions; ($p<0.05$).

[f] Significantly different from corresponding K-H solution; ($p<0.05$).

[g] Significantly different from K-H solutions at FREQ 60,27C and FREQ 60,37C; ($p<0.05$).

[h] Significantly different from K-H solution at FREQ 60,37C; ($p<0.05$).

Table 15. Summary of the effects of shock nomogenate, frequency and temperature on time to maximum velocity of relaxation (TMVR)

	Normalized Relative Load (P/Po) [a]				
	0Po	0.2Po	0.4Po	0.6P0	0.8P0
FREQ 12, 27C (n=8) [b]					
	TMVR (msec) [c]				
Shock Peak	562 [d] ± 79	585 ± 46	582 ± 45	573 ± 54	555 ± 66
Krebs-Henseleit	573 [e] ± 54	562 [e] ± 51	564 [e] ± 49	563 [e] ± 62	544 [e] ± 68
FREQ 12, 37C (n=6)					
Shock Peak	351 [f] ± 32	345 [f] ± 28	340 [f] ± 26	334 [f] ± 27	326 [f] ± 30
Krebs-Henseleit	349 [g] ± 30	342 [g] ± 29	333 [g] ± 32	323 [g] ± 34	313 [g] ± 27

FREQ 60, 27C (n=11)					
Shock Peak	500 ± 33	485 ± 43	472 ± 48	459 ± 47	442 ± 43
Krebs-Henseleit	460[h] ± 44	446[h] ± 46	441[h] ± 45	435[h] ± 41	420 ± 40
FREQ 60, 37C (n=10)					
Shock Peak	311 ± 28	307 ± 29	302 ± 30	296 ± 30	288 ± 30
Krebs-Henseleit	298 ± 42	295 ± 37	292 ± 34	289 ± 35	285 ± 40

[a] Calculated as 0.2 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded time versus afterload data were fitted to third order polynomial equations and time then extrapolated from the equation at 0.2 increments of Po.

[d] All values expressed as mean ± S.D.

[e] Significantly different from all other K-H solutions; ($p<0.05$).

[f] Significantly different from corresponding Krebs-Henseleit; ($p<0.05$).

[g] Significantly different from K-H solution at FREQ 60,27C and FREQ 60,37C; ($p<0.05$).

[h] Significantly different from K-H solution at FREQ 60,37C; ($p<0.05$).

Table 16. Summary of the effects of shock homogenate, frequency and temperature on duration of contraction (DUR)

	Normalized Relative Load (P/Po) [a]				
	0	0.2Po	0.4Po	0.6Po	0.8Po
FREQ 12, 27C (n=8)[b]					
	DUR (msec)[c]				
Shock Peak	804[d] ± 71	675 ± 41	637 ± 44	638 ± 61	625 ± 73
Krebs-Henseleit[e]	789 ± 52	649 ± 45	614 ± 46	623 ± 57	609 ± 67
FREQ 12, 37C (n=6)					
Shock Peak	552[f] ± 74	440[f] ± 49	402[f] ± 56	398[f] ± 53	388 ± 45
Krebs-Henseleit	557 ± 64	440 ± 50	394 ± 52	385 ± 54	378 ± 49

FREQ 60, 27C (n=11)					
Shock Peak	698 ± 63	561 ± 66	516 ± 56	512 ± 48	498 ± 51
Krebs-Henseleit[e]	647 ± 84	537 ± 65	493 ± 52	483 ± 52	475 ± 49
FREQ 60, 37C (n=10)					
Shock Peak	427 ± 48	378 ± 44	356 ± 43	347 ± 44	359 ± 47
Krebs-Henseleit[e]	430 ± 70	362 ± 51	335 ± 46	332 ± 47	333 ± 46

[a] Calculated as 0.2 increments of Po for respective solutions normalized for cross-sectional area.

[b] FREQ = frequency (12 or 60/minute); C = temperature (27 or 37 C).

[c] Calculated as follows: recorded time versus afterload data were fitted to third order polynomial equations and time then extrapolated from the equation at 0.2 increments of Po.

[d] All values expressed as mean ± S.D.

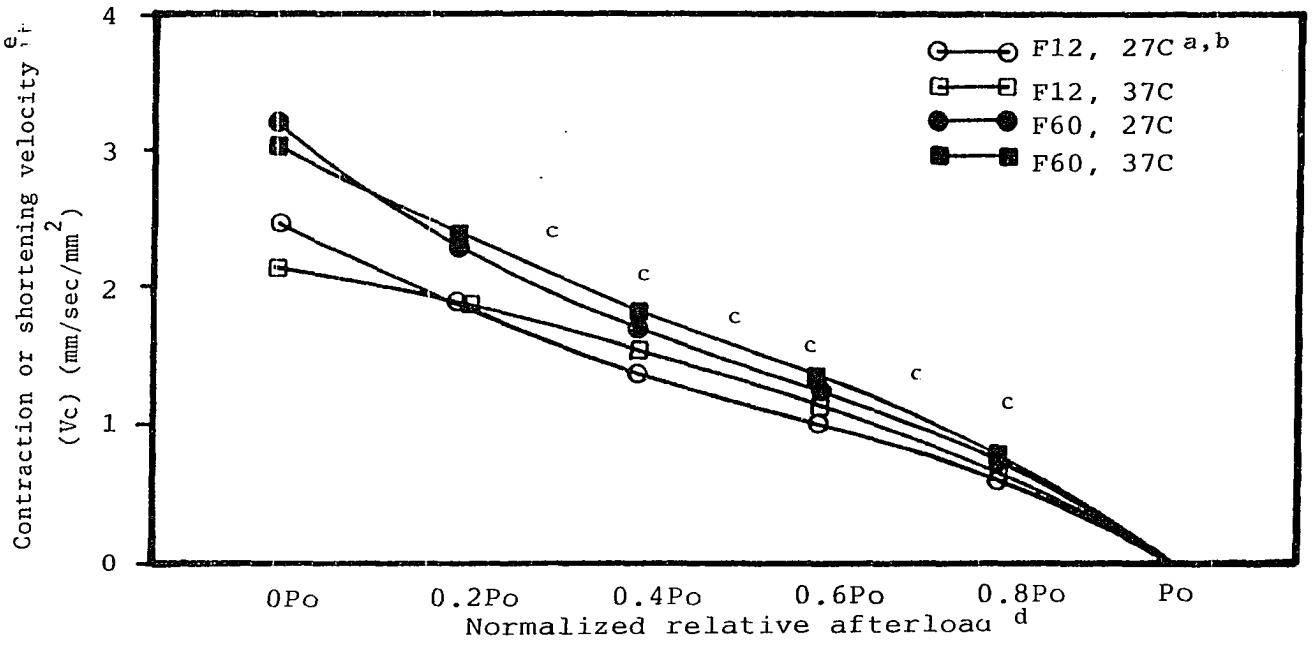
[e] All Krebs-Henseleit means significantly different from corresponding mean at other temperatures and frequencies; ($p<0.05$).

[f] Significantly different from corresponding K-H solution; ($p<0.05$).

given, but the correlation coefficient of the fit was almost always in excess of 0.95 for the velocity curves, latency (Lat), time to maximum velocity of contraction (TMVC), and duration (Dur). The various time data presented in Tables 12 through 16 have been reported only for 0.2 increments of Po because the shape of the curves did not exhibit marked changes between those points.

Effects of frequency and temperature on the isotonic contraction of canine papillary muscles measured in Krebs-Henseleit solution

Figures 27 through 30 illustrate the effects of combinations of temperature and frequency on muscle function measured in the Krebs-Henseleit solution. The changes in maximum load (Po) are shown in Figure 27. The temperature effects are consistent with those observed in the previous section for the isometric preparation and confirm those reported in the literature by Parmley and Sonnenblick (1969), Mattiazzi and Nilsson (1976), and Frist et al. (1978). Decreasing the temperature produced a significant positive inotropic effect at both frequencies. Further, the temperature effect is more prominent at low frequency. A significant positive inotropic effect was also observed when the frequency was increased at 37 C but not at 27 C. Faster delivery of the stimulus would be expected to mobilize more calcium per time. Likewise, increasing the temperature might be expected to increase both transsarcolemmal calcium flux and intracellular ion mobility and raise the free calcium necessary for contraction. However, increasing the temperature should also increase efficiency of the metabolic machinery which lowers sarcoplasmic calcium levels via calcium reuptake by the sarcoplasmic reticulum. The lower maximum load at 37 C is evidence



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[a] Mean response at each afterload.

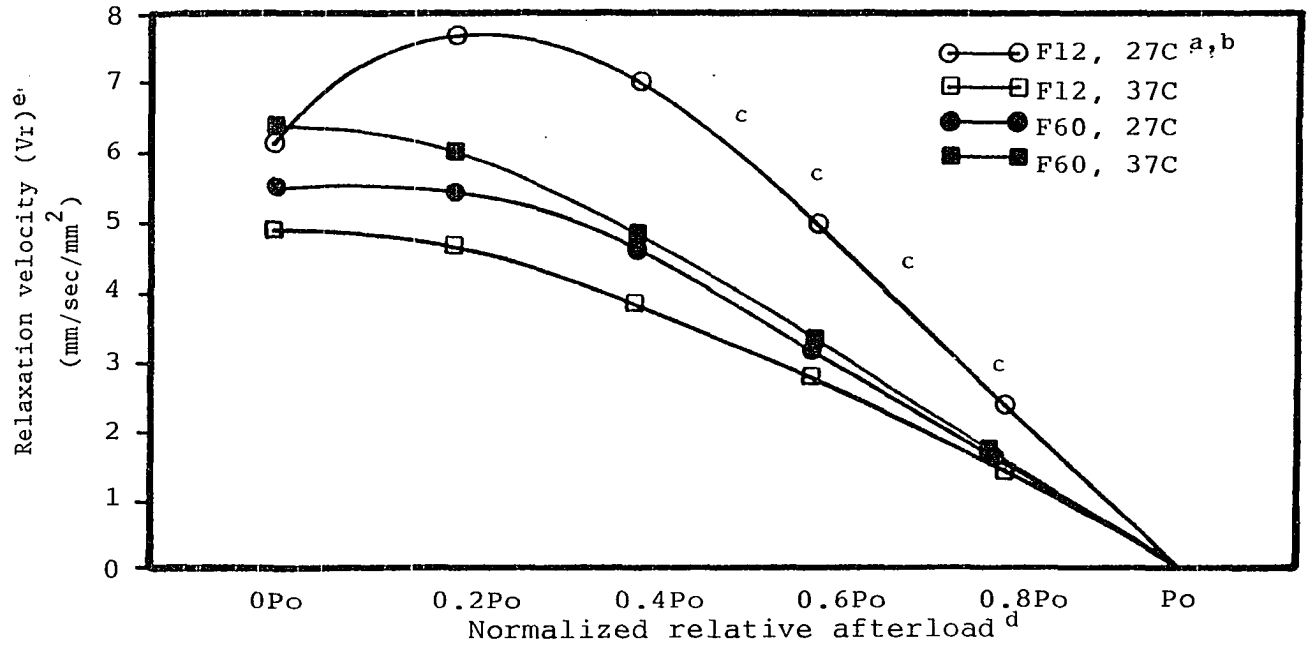
[b] F = frequency (12 or 60/minute); C = temperature (27 or 37C).

[c] Significant effect of frequency at 27C from 0.3-0.8Po; ($p<0.05$).

[d] Calculated as 0.1 increments of Po for each respective solution and normalized for cross-sectional area.

[e] Calculated as follows: recorded velocity versus afterload data were fitted to a third order polynomial equation and velocity then extrapolated from the equation at 0.1 increments of Po.

Figure 28. Relationship between afterload and contraction or shortening velocity at combinations of frequency and temperature



[a] Mean response at each afterload.

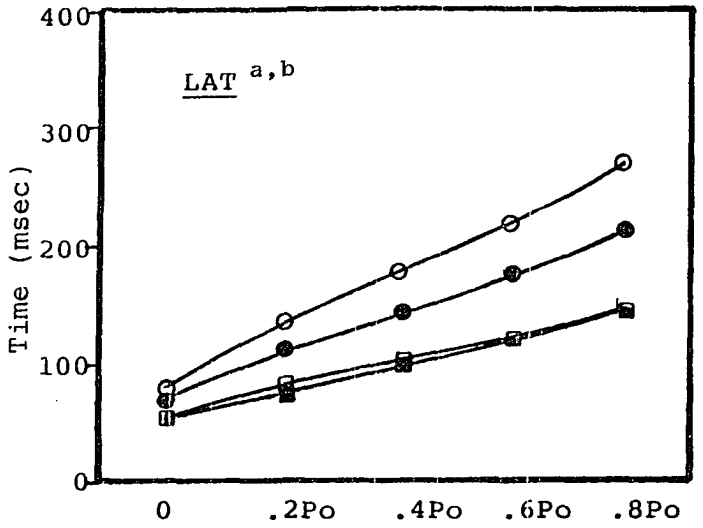
[b] F = frequency (12 or 60/minute); C = temperature (27 or 37C).

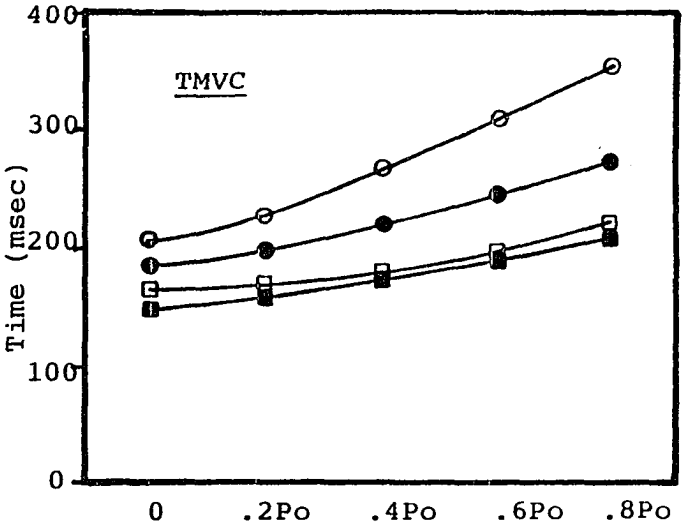
[c] Significant effect of frequency at 27C from 0.5-0.8Po; ($p<0.05$).

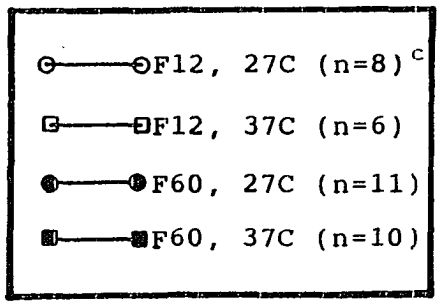
[d] Calculated as 0.1 increments of Po for each respective solution and normalized for cross-sectional area.

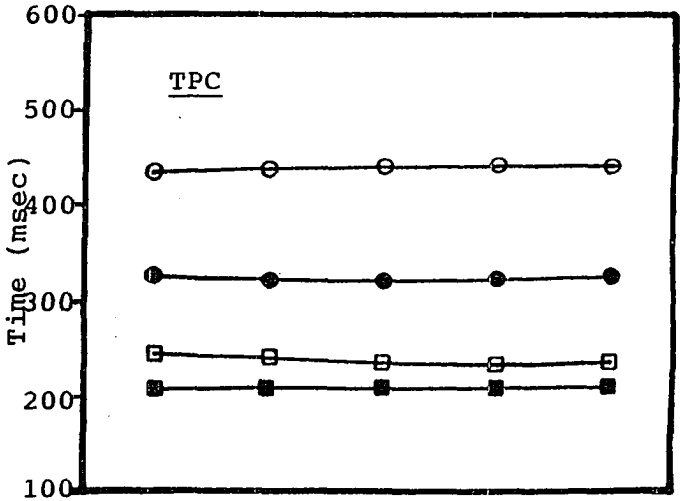
[e] Calculated as follows: recorded velocity versus afterload data were fitted to a third order polynomial equation and velocity then extrapolated from the equation at 0.1 increments of Po.

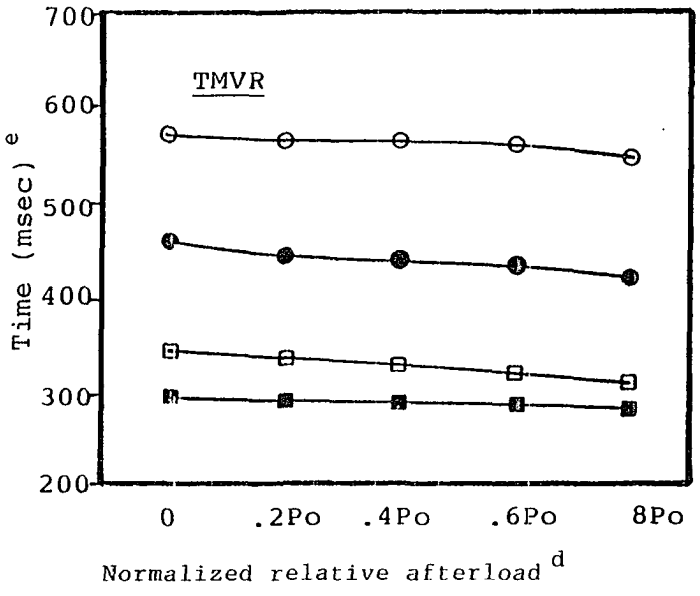
Figure 29. Relationship between afterload and relaxation velocity at combinations of frequency and temperature measured in Krebs-Henseleit

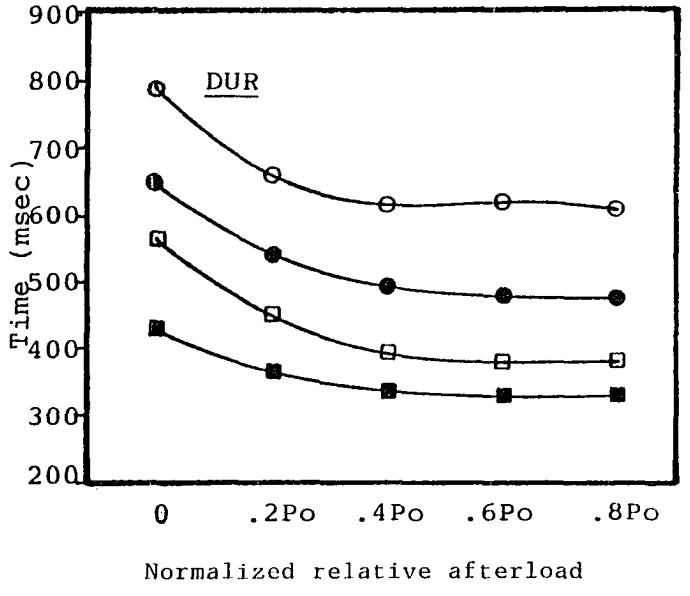

LAT a,b
Time (msec)
400
300
200
100
0
0
.2Po
.4Po
.6Po
.8Po


TMVC
Time (msec)
400
300
200
100
0
0
.2Po
.4Po
.6Po
.8Po


F12, 27C (n=8) c
F12, 37C (n=6)
F60, 27C (n=11)
F60, 37C (n=10)


TPC
Time (msec)
600
500
400
300
200
100





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[a] Mean response at each afterload.

[b] Statistical significance has not been shown as the differences have been reported in Tables 12-16. Most means were significantly different.

[c] F = frequency (12 or 60/minute); C = temperature (27 or 37C).

[d] Calculated as 0.2 increments of Po for each respective solution.

[e] Calculated as follows: recorded time versus afterload data were fitted to a third order polynomial equation and velocity then extrapolated from the equation at 0.2 increments of Po.

Figure 30. Summary of frequency and temperature on the time to various phases of the isotonic contraction recorded in Krebs-Henseleit

which supports the dominance of the later process at 37 C. Tension developed or the number of cross bridges formed is a direct function of the cytoplasmic calcium concentration which is lowered as temperature is increased. Temperature and frequency are inversely related in the potential of each factor to produce an inotropic effect on the canine muscle preparation.

The relationships between afterload and both contraction and relaxation velocity at the combination of frequency and temperature measured in Krebs-Henseleit solution are shown in Figures 28 and 29. The fastest contraction velocity was observed at both temperatures when the frequency was 60 per minute. However, the difference was only significant over the middle range of afterload. Although little significant difference between treatments was observed, the contractile velocity at a constant frequency was usually fastest at low temperature at afterloads less than 0.2Po. As the afterload weights were increased in excess of 0.2Po, the velocity tended to be faster at high temperature. Whether this frequent observation is real and not artifact or if this relates to differences in muscle stiffness, calcium release, or calcium reuptake is speculative at best.

A significant effect of frequency was the only observed difference in relaxation velocity, as shown in Figure 29. Considerable variation in maximum relaxation velocity over a range of approximately 1-20 mm/sec was observed. Maximum velocity was observed at approximately 0.2-0.3Po. This was a more consistent observation at lower temperature. Peak velocity at 0.2-0.3Po was also observed in the experiments to examine shock plasma and further confirms the observations of Strauer (1973) and

Strobeck et al. (1975) with isotonic preparations of feline papillary muscle at temperatures of 24 and 29 C, respectively. Strobeck et al. have suggested that this phenomenon may be caused by increased stress placed on those cross-bridges which are intact during the relaxation process. Fewer bridges are being formed and the afterload would tend to break those remaining at a faster rate. A maximum rate at any afterload would be a function load, extent of activation, and the rate of calcium sequestration.

Relaxation velocity was fastest for all conditions at low temperature and frequency but slowest when the temperature was increased. This observation would seem to be contradictory to a faster reuptake of calcium at higher temperature. If the responses in Figure 29 for both temperatures at a frequency of 60/minute are compared, a slightly faster relaxation velocity occurred when temperature was increased. It is possible that the curve for F12, 27C may represent an artifact due to a proportionately greater number of muscles in that group which exhibited very high relaxation velocities. It should be reemphasized that each muscle could not always be tested at all combinations of temperature and frequency. No other explanation for this observation is apparent at this time. This study was conducted to evaluate the interaction of temperature, frequency, and the shock peak and not specifically temperature or frequency. Primary comparison of these factors was subsequently accomplished by normalizing the results by expressing them as a percent change from the paired response in Krebs-Henseleit solution.

The effects on the various time intervals are shown in Figure 30.

Latency was shortened at constant frequency over the range of afterloads tested when the temperature was increased. In contrast, increasing the frequency shortened latency only at 27 C . Latency is a measure of the time from delivery of the stimulus until sufficient calcium has been mobilized for contraction to begin. Therefore, a faster frequency or a lower temperature would help to maintain the calcium concentration closer to the critical threshold and shorten latency. The other times to the various phases of the contraction cycle were also temperature sensitive over the entire range of afterloads. TMVC, TPC, TMVR, and duration were all shortened by raising the temperature to 37 C. An increase in frequency also significantly shortened all parameters at both temperatures except for TMVC at 27 C. Temperature had a more pronounced effect than frequency on altering time in the contractile cycle.

Latency, TMVC, and duration all appear to be load dependent. As the amount of afterload is increased, a proportionately longer time would be required to develop sufficient tension for muscle shortening to begin. Therefore, both load dependence of latency and TMVC (which is a measurement of shortening velocity) would be expected. Gravity acts on the afterload weight hung on the opposite end of the isotonic muscle lever. This force aids in the relaxation process by exerting a shearing force which tends to break cross-bridges and restore the resting sarcomere length more rapidly. Muscles do not shorten as much with heavier afterload which minimizes the number of potential cross-bridges which may be formed during the relaxation or lengthening phase of the cycle. Therefore, both of these factors would be expected to contribute

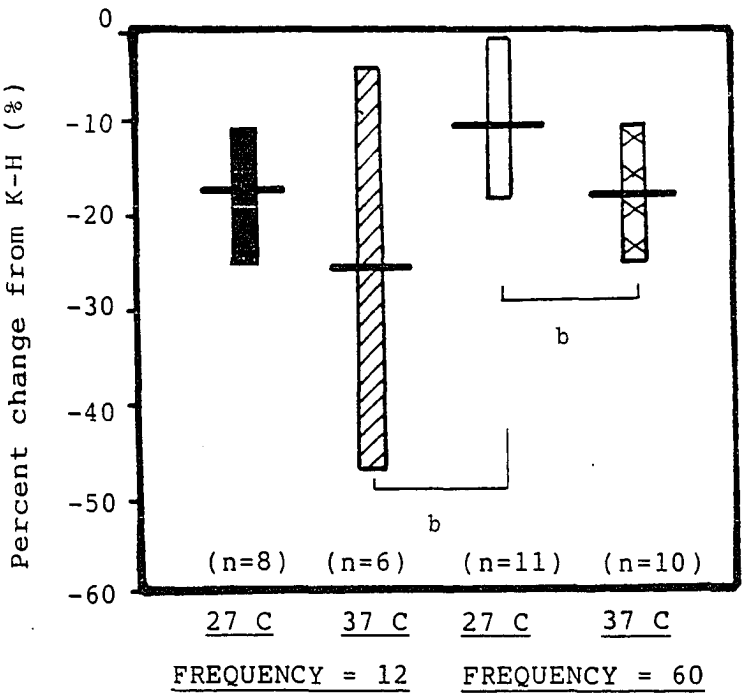
to a decrease in the duration of the contraction. The time to the peak of the contraction (TPC) does not vary with the relative amount of afterload and appears to be load independent. TMVR also is relatively constant over the range of afterloads, although a subtle trend to decrease with increasing afterload is shown in Figure 30.

Effects of frequency and temperature on the response of the papillary muscles to the shock peak The interaction of shock peak, frequency, and temperature on maximum load has been summarized in Figure 27. The shock peak caused a significant depression of maximum load at all combinations of temperature and frequency except at 37 C and a frequency of 12/minute (F12,37C). Although the same isotonic preparation was utilized in the bioassay of shock plasma, no significant depression of maximum load was observed in those studies. However, depression of peak isometric tension was observed in the studies conducted to determine the presence of the shock fraction in the chromatographic separation. Whole plasma may contain other substances expected to have positive inotropic effects on the muscle. Any such influence was removed by the chromatographic separation of the ultrafiltered homogenate. The isotonic muscle preparation must develop isometric tension equal to the afterload before shortening can occur. Shortening requires formation of new cross-bridges as the sarcomere length decreases and is more energy costly. The concentration of the depressant in both the isometric and present isotonic study were theoretically the same. Therefore, it is possible that the isotonic preparation may be more sensitive to the depressant.

The presence of the depressant factor was confirmed by the observed

depression maximum load caused by the shock homogenate peak but not the control homogenate peak. In order to determine if there were differences in the relative amount of depression produced by combinations of temperature or frequency, the recorded effect of the shock peak on maximum load at the various conditions for each experiment was expressed as a percent change from the recorded response to Krebs-Henseleit solution and statistically compared. These results are summarized in Figure 31. Despite a lack of statistical difference between the two solutions, the largest percent depression (approximately 25%) was observed at low frequency and the higher temperature (F12,37C). An increase in frequency at a given temperature did not cause any significantly different response, but a trend towards less depression was observed.

The interactions of frequency, temperature, and shock peak on contraction and relaxation velocity have been summarized in Tables 11 and 12. The shock peak produced significant depression of contraction or shortening velocity primarily at low frequency for a constant temperature. However, depression was only observed at afterloads less than one half maximum load. This observation would appear to be contrary to what might have been anticipated. Intuitively, diminished function caused by the shock peak via the undefined mechanism would be expected to have a more pronounced effect at heavier afterloads. More cross-bridges would need to be formed and the energy expenditure increased. Therefore, these results may reflect some artifact created by expressing data which differ in magnitude between individual muscles as mean $\pm$ S.D. Relaxation velocity, which is summarized in Table 11,



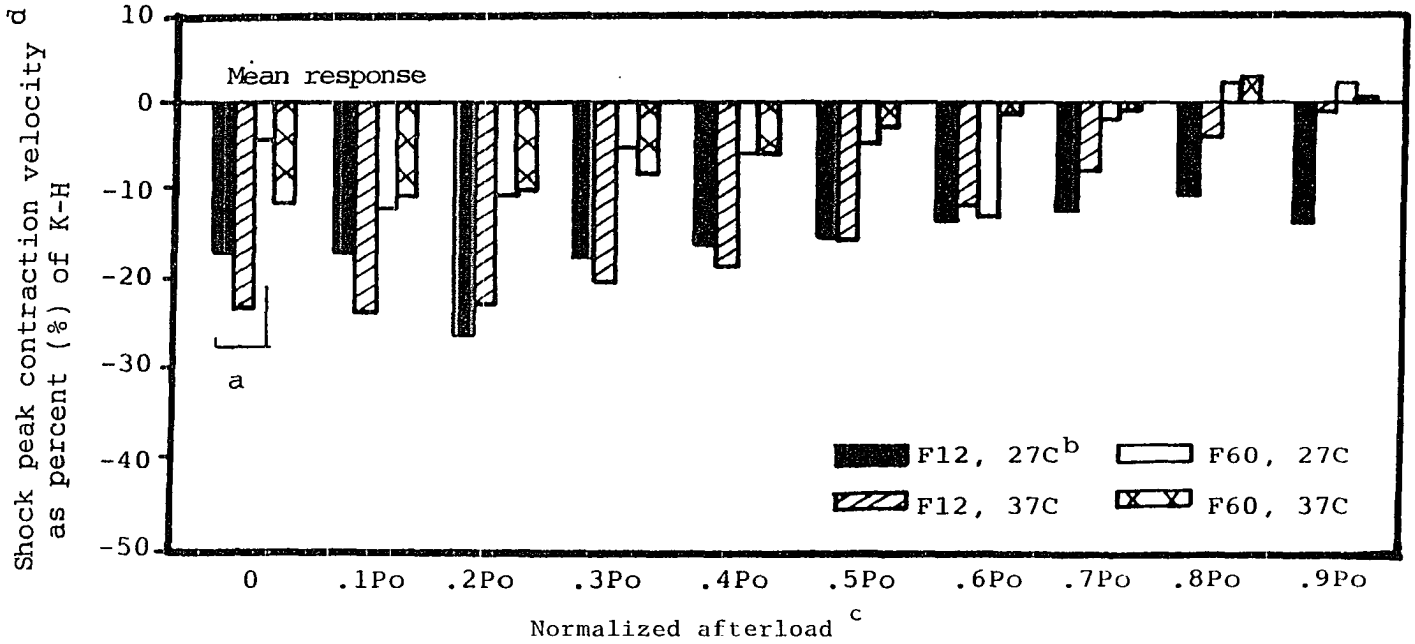
[a] All values expressed as mean ± S.D.
[b] Comparisons are significantly different; ($p<0.05$).

Figure 31. Effects of frequency and temperature on the relative depression of maximum load produced by the shock peak

was significantly depressed over the entire range of afterloads but only at low frequency.

The velocities have been expressed as percent change from K-H solution in Figures 32 and 33. A frequency induced relative depression of contraction velocity at 27C with no additional afterload was the only significant observation. If the magnitude of depression of contraction velocity at each condition is compared, depression at low frequency was twice that observed at the faster frequency over the range of afterloads. A trend towards less depression with increasing afterload may also be observed in Figure 32.

Relative changes in relaxation velocity are presented in Figure 33. The predominant effect was that of frequency. At a constant temperature the amount of depression at a frequency of 12/minute was double that at 60/minute. Higher temperature also tended to cause more depression, especially at a frequency of 60/minute. There was comparatively little difference in relaxation velocity between the shock peak and Krebs-Henseleit at F60,27C. Under those conditions, a theoretically higher concentration of free calcium over time would be anticipated due to increased calcium mobilization induced by frequency and an anticipated low temperature induced inhibition of calcium reuptake by the metabolic dependent process of the sarcoplasmic reticulum. This contrasts with the more pronounced effect at F12,37C where less calcium would be available over time. The depression effect on velocity appears to be antagonized at experimental conditions which raise the cytoplasmic calcium levels. This further confirms the observations of Lefer and Rovetto (1970) that supplemental exogenous calcium can reverse the



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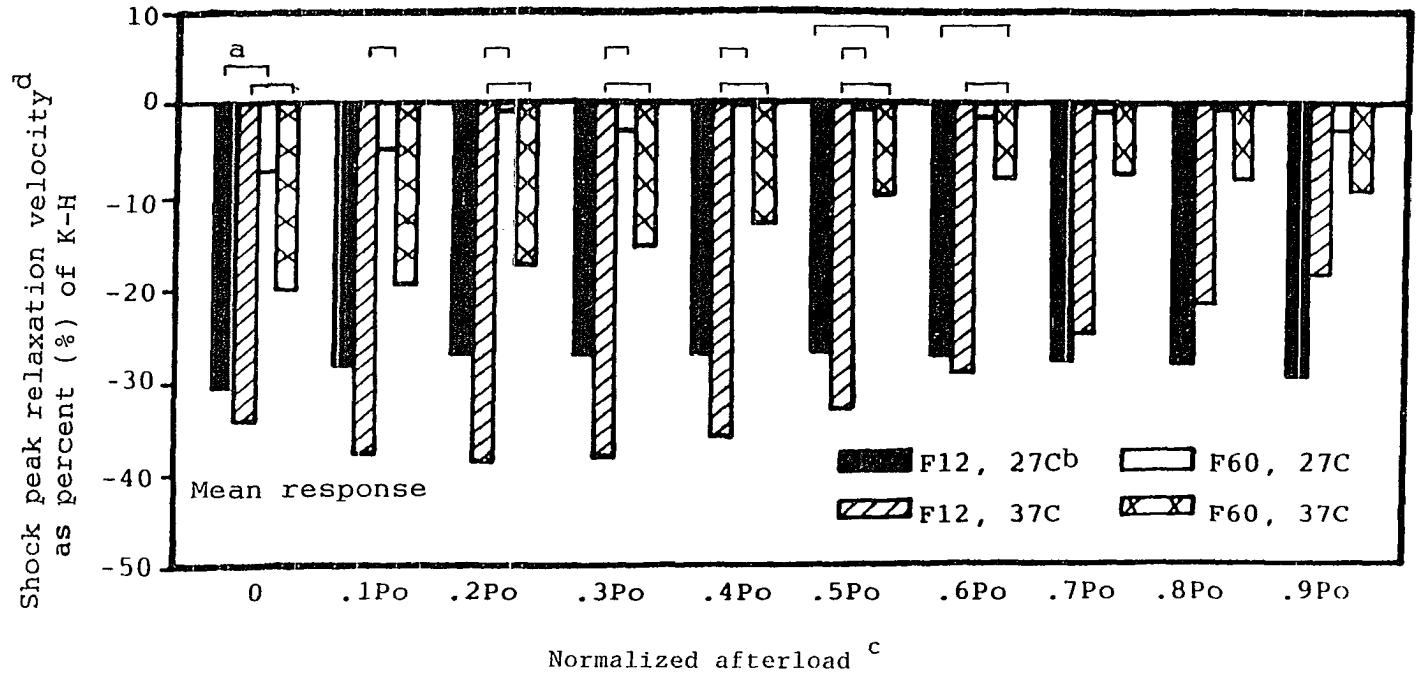
a Significant difference between indicated means; ($p<0.05$).
b F = frequency (12 or 60/minute); C = temperature (27 or 37C).
c Calculated as 0.1 increments of Po for respective solutions.
d Recorded velocity data fitted to a third order polynomial, the velocity was extrapolated from the fit at 0.1 increments of Po, and then expressed as percent change from corresponding velocity for post-peak K-H solution.

Figure 32. Relative change in shortening or contraction velocity due to the shock peak and combinations of frequency and temperature



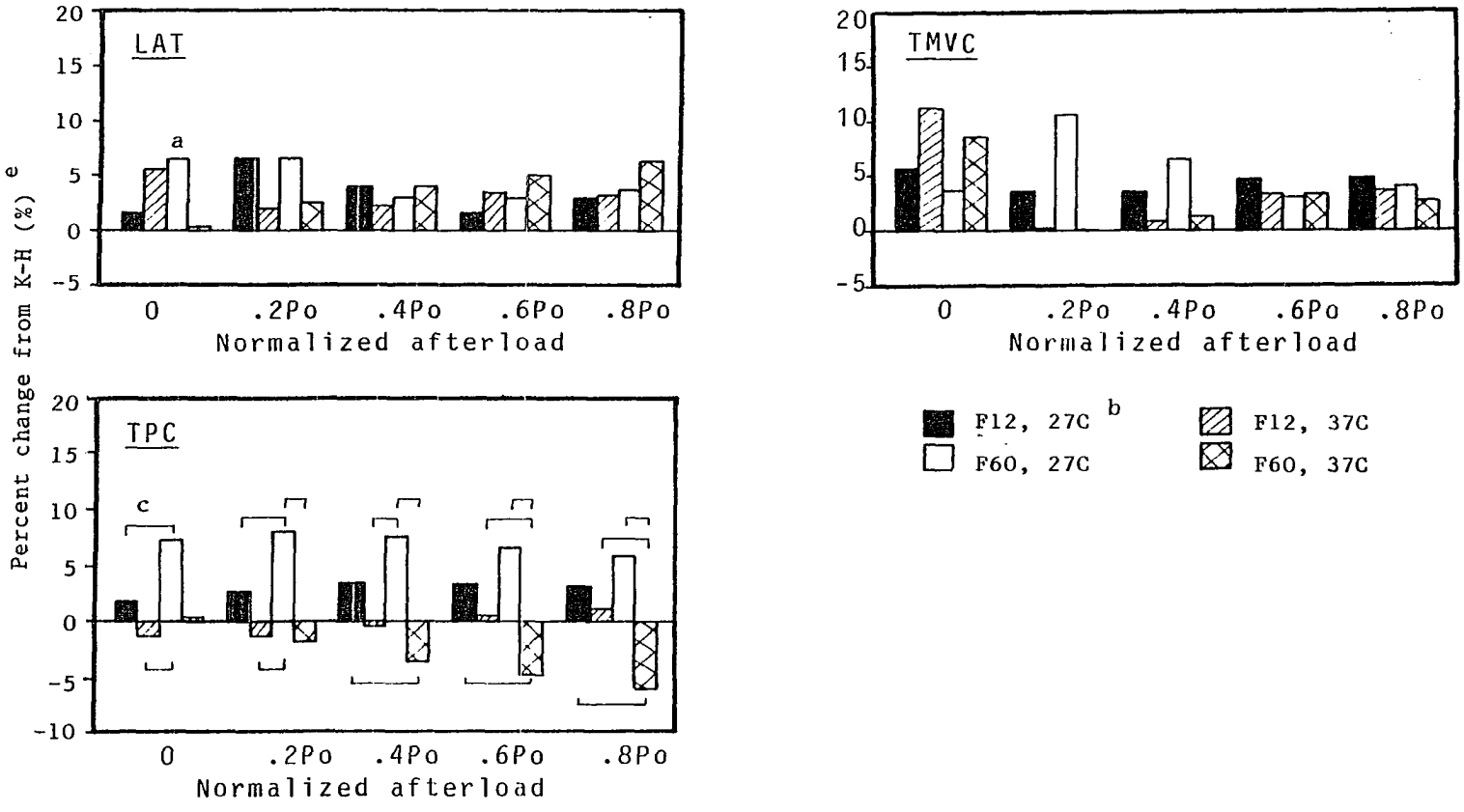
[a] Significant difference between indicated means; ($p<0.05$).
[b] F = frequency (12 or 60/minute); C = temperature (27 or 37C).
[c] Calculated as 0.1 increments of Po for respective solutions.
[d] Recorded velocity data fitted to a third order polynomial, the velocity was extrapolated from the fit at 0.1 increments of Po, and then expressed as percent change from corresponding velocity for post-peak K-H solution.

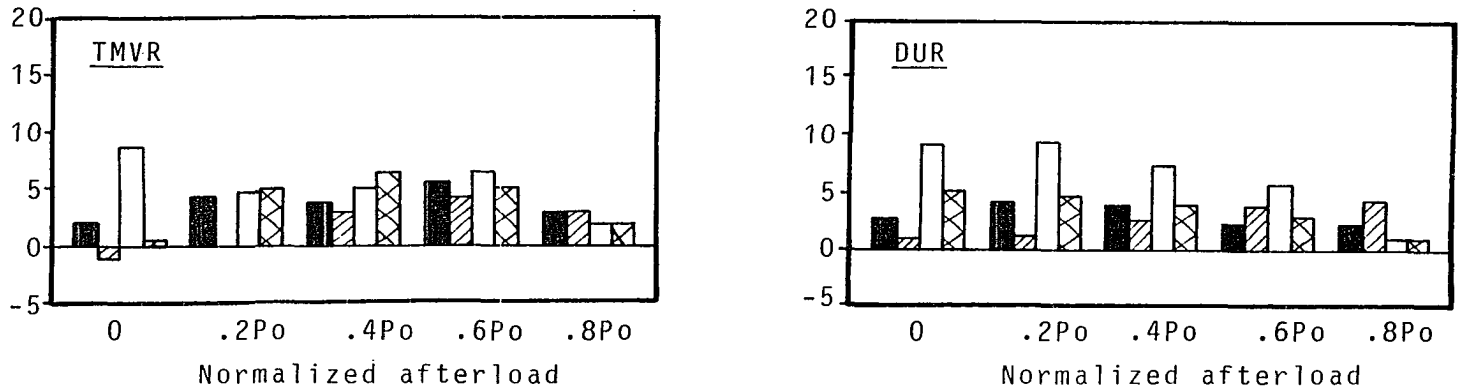
Figure 33. Relative change in relaxation velocity due to shock peak and combinations of frequency and temperature

depression caused by MDF. When the magnitude of depression for maximum load and contraction and relaxation velocity are compared for any given condition, the observation that relaxation velocity is the primary parameter affected by the depressant peak is again confirmed.

The relative changes calculated for the various time intervals have been summarized in Figure 34. Prolongation of the LAT, TMVC, TMVR, and DUR was observed over the entire range of afterloads. However, there were no significant differences between the effects of combinations of frequency and temperature on the response to the shock peak. A trend towards a slower TMVR at higher frequency, especially at 27 C, was the only consistent pattern observed.

Relative change in the TPC produced by the shock peak was significantly affected by combinations of temperature and frequency. The shock peak caused a prolongation of the TPC at 27 C, but this effect was antagonized by raising the temperature to 37 C. Effects of temperature were most pronounced at the higher frequency of 60/minute. The effects of cooling on prolonging the TPC of the muscles tested in Krebs-Henseleit solution are illustrated in Figure 30. Cooling would be expected to slow the mobilization of calcium for contraction and to inhibit the enzymatic processes for formation and cleavage of cross-bridges. The prolonging of the TPC at F60, 27C by the shock peak could imply that alteration of either of these schemes is a possible mechanism for the depressant. Shortening the TPC at F60, 37C must also be reconciled. Elevating the temperature may sufficiently activate a key enzymatic process or slow the rate of calcium release and mask any inhibition of the depressant. This hypothesis would not seem likely


LAT
TMVC
TPC
a
c
20
15
10
5
0
-5
-10
0
.2Po
.4Po
.6Po
.8Po
Normalized afterload
Percent change from K-H (%) e
F12, 27C b
F60, 27C
F12, 37C
F60, 37C



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[a] Mean response per treatment and afterload.
[b] F = frequency (12 or 60/minute); C = temperature (27 or 37 C).
[c] Significant difference between indicated means; ($p<0.05$).
[d] Calculated as 0.2 increments of Po for respective solutions.
[e] Calculated as follows: recorded time data were fitted to a third order polynomial, the specific time was then extrapolated from the fit at 0.2 increments of Po, and then expressed as percent change from the corresponding time calculated for post-peak K-H solution.

Figure 34. Relative change in the various time phases due to the shock peak and combinations of frequency and temperature

because changes in the TPC must be reconciled with a quantitative depressant effect on cardiac muscle. Greater relative depression was observed at 37 C. If maximum shortening were reached earlier in a muscle but the degree of shortening was quantitatively less, then fewer cross-bridges would be formed and less work would be done. In this later case, cooling would afford some protection by prolonging the pulse of available calcium.

The shock peak caused further prolongation of the TPC at 27 C when frequency was increased. At 37 C the frequency effect is reversed and a shortened TPC was observed. Neither temperature nor frequency influenced the TPC with increasing afterload of muscles tested in Krebs-Henseleit, as shown in Figure 30. However, the shock peak caused a progressive reduction in the TPC with increasing afterload at F60, 37C. The interaction of temperature and frequency alters the effects of the shock peak on TPC.

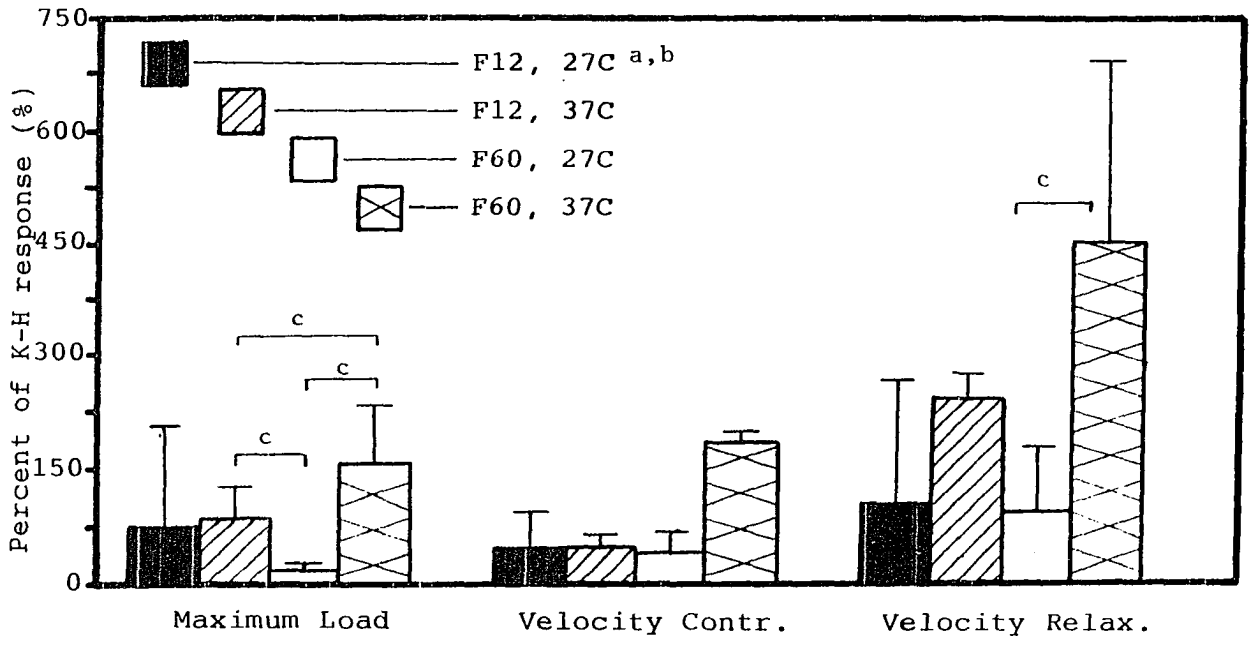
Effects of isoproterenol on muscle function at different frequencies and temperatures Isoproterenol was added to the Krebs-Henseleit solution in the muscle chamber at the conclusion of all other testing and the response recorded. Final concentration in the chamber fluid was 10^{-6} M. Complete afterload curves were done in seven of the muscles. Determinations of maximum load (Po) and the response with no afterload were made in the remaining muscles. There were insufficient numbers in all groups representing combinations of temperature and frequency for statistical comparisons. Selected data have been summarized in Table 17 and the relative change in the measured parameters produced by isoproterenol is shown in Figures 35 and 36.

Table 17. Summary of the effects of isoproterenol at different frequencies and temperatures on isotonic muscle function

PARAMETER	F12,27C[a,b,c] ISOP	F12,27C K-H	F12,37C ISOP	F12,37C K-H	F60,27C ISOP	F60,27C K-H	F60,37C ISOP	F60,37C K-H
Max. Load (g/mm^2)	3.23[d] ± 2.51	2.34 ± 2.49	1.11[e] ± 0.22	0.63 ± 0.25	2.80[e] ± 0.78	2.34 ± 0.58	1.85[e] ± 0.35	0.80 ± 0.31
Vc ($mm/sec/mm^2$)	5.08 ± 2.45	3.89 ± 2.69	3.60[e] ± 1.19	2.23 ± 0.95	6.97[e] ± 5.89	4.54 ± 3.24	4.46[e] ± 1.42	1.90 ± 1.33
Vr ($mm/sec/mm^2$)	19.69 ± 14.41	14.18 ± 15.49	13.85 ± 8.95	4.60 ± 1.56	10.94 ± 6.31	6.03 ± 3.41	17.19[e] ± 2.60	3.57 ± 1.81
LAT (msec)	78 ± 11	100 ± 38	50 ± 8	62 ± 5	59 ± 17	79 ± 15	42[e] ± 14	63 ± 12
TMVC (msec)	166 ± 9	262 ± 77	131 ± 17	138 ± 7	148 ± 16	175 ± 30	119[e] ± 28	158 ± 32
TPC (msec)	455 ± 30	479 ± 21	242 ± 18	247 ± 30	306 ± 52	315 ± 48	179 ± 22	198 ± 25

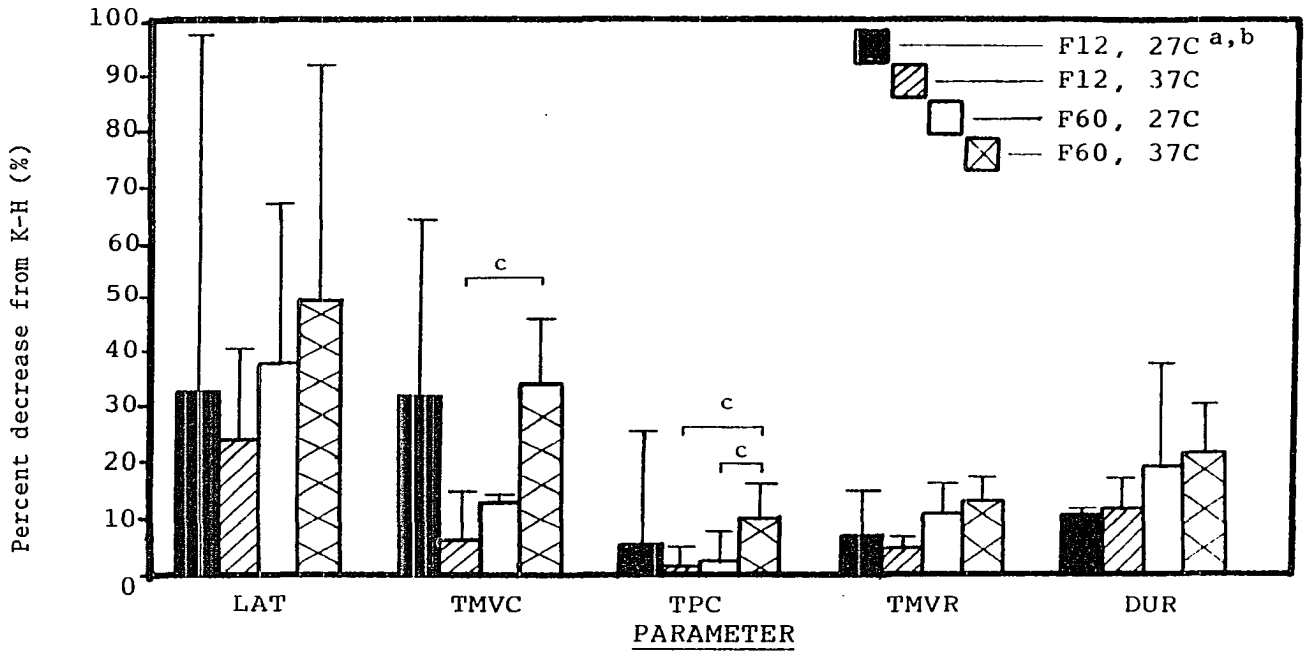
TMVR (msec)	587 ± 23	631 ± 33	518[e] ± 23	554 ± 30	451[e] ± 41	479 ± 51	505[e] ± 89	252 ± 98
DUR (msec)	772 ± 71	863 ± 73	457[e] ± 56	509 ± 36	633 ± 153	660 ± 99	554[e] ± 51	429 ± 52
(n)[f]	2		3		3		5	

[a] All responses except for maximum load were recorded with no afterload.
[b] Parameters were previously defined in Figure 3.
[c] F = frequency (12 or 60/min); C = temperature (27 or 37 C).
[d] All values expressed as mean ± S.D.
[e] Significant effect of isoproterenol; ($p<0.05$).
[f] No statistics applied for groups with $n<3$.



[a] All values expressed as mean ± S.D.
[b] F = frequency (12 or 60/minute); C = temperature (27 or 37C).
[c] Significant difference between indicated pairs; ($p<0.05$).

Figure 35. Relative changes in maximum load and contraction and relaxation velocities in response to frequency, temperature, and isoproterenol (10^{-6} M)



[a] All values expressed as mean ± S.D.
[b] F = frequency (12 or 60/minute); C = temperature (27 or 37C).
[c] Significant difference between indicated pairs; ($p<0.05$).

Figure 36. Relative changes in latency times in response to frequency, temperature, and isoproterenol (10^{-6} M)

Maximum load (Po) and both contraction (shortening) velocity and relaxation velocity with no afterload added to the muscle were all increased by isoproterenol. Increasing the frequency appeared to have little effect on either contraction or relaxation velocity, but maximum load was reduced at 27 C. In contrast, a more rapid stimulus appeared to have a pronounced effect on maximum load and both velocities at 37C. Isoproterenol had the greatest effect at the combination of higher temperature and frequency. Further, when the relative changes in velocity are compared for all combinations of temperature and frequency, the magnitude of change in relaxation velocity is greater than contraction velocity.

The effects on the time to various events in the isotonic contraction cycle of isoproterenol relative to K-H solution are shown in Figure 36. Considerable variation and small sample numbers precluded any definitive conclusions. However, isoproterenol did shorten the respective times in the experiments. It should be noted that even these modest changes may represent a decrease of almost 50 msec and would be expected to have a marked effect on the availability of calcium for contraction. Isoproterenol did not appear to greatly change the time to peak shortening (TPC) at any combination of temperature or frequency, although the considerable difference in the absolute TPC can be observed in Table 17. The dominant effect at all combinations was to decrease the time to the onset of contraction (LAT) by 25-50%. This would confirm that these preparations can respond to catecholamine stimulation after as much as eight hours of <u>in vitro</u> experimentation. There is insufficient data to determine whether temperature or frequency altered

the response to isoproterenol. However, the greatest decrease in all times was also observed at the combination of higher temperature and frequency.

A consistent observation following administration of isoproterenol was a rapid and often irreversible deterioration of the papillary muscles which was most evident when the preparation was tested at primarily 37 C but also at 60 stimuli/minute. Neither repeated washing with fresh Krebs-Henseleit solution nor altering the temperature or frequency prevented the loss of function. Isoproterenol increases metabolism via β-adrenergic stimulation. The added demands on oxygen diffusion to the muscle core probably exceeded the availability to deliver oxygen and probably resulted in permanent damage due to a hypoxic core in the muscle.

Evaluation of the papillary muscles with isoproterenol did confirm that the muscles were responsive to a positive inotropic agent despite repeated changes of solutions and alterations in frequency and temperature over 6-10 hours of experimentation. Changes in muscle function over time would be anticipated. However, deterioration over time was compensated for by always referencing the response of either the control or shock peak to corresponding measurements made in a subsequent Krebs-Henseleit solution.

This study was conducted to evaluate the influence of temperature and frequency upon the response of isotonically contracting canine papillary muscles to a chromatographic fraction of a pancreatic homogenate previously subjected to shock or control conditions. The control homogenate peak did not alter any of the measured parameters

except to cause some improved function relative to the response in Krebs-Henseleit solution. The shock homogenate peak significantly depressed maximum load, both contraction and relaxation velocity, and the time to peak contraction (TPC). These effects appear to be influenced by the temperature and frequency of the muscle preparation. The results are consistent with a more pronounced effect of the shock peak at physiological temperature. A similar observation was reported by Williams et al. (1969). They observed depression of peak developed isometric tension and the maximum rate of tension development at 37 C but not at 30 C in response to dialysates of shock plasma. The time to peak tension was not altered in their study. Increasing the frequency of the muscle preparation in the present study appeared to antagonize the depressant effects on velocity and the TPC but not necessarily maximum load. This study reinforces the need to evaluate potential temperature and frequency effects on in vitro bioassay system used to evaluate inotropic agents. Further, it is possible that previously reported failures to detect an endogenous depressant factor with in vitro preparations may have been obscured by the inappropriate combination of these factors.

The present study was conducted over a three month period but was not begun until six months after the homogenates had been collected and stored. Loss of activity with storage was not evaluated, yet measurable depressant activity was still possible. There were differences in the magnitude of depression observed in the numerous studies of Lefer and colleagues. Lefer typically reported 50-70% depression of peak developed isometric tension, the primary criteria for assessing the

depressant effect of myocardial depressant factor. In contrast, maximum load was depressed by 15-25% in these studies. A concentration effect, differences in the preparation of the pancreatic specimens, use of different species for the bioassay system, use of isometric versus an isotonic preparation, or even the age of the preparation could account for these differences.

It is not possible to identify the depressant factor in the present study as Lefer's myocardial depressant factor. However, there is no reason to believe that it may not be MDF. It was pancreatic in origin, prepared and processed by similar techniques, has a molecular weight between 500-1000, and depresses isolated isotonically contracting canine myocardium.

GENERAL DISCUSSION AND CONCLUSIONS

Methods comparable to those employed by Lefer and colleagues were used to evaluate fresh whole shock plasma and pancreatic homogenates for the presence of a myocardial depressant. Although the canine has been utilized as a source for Lefer's myocardial depressant factor, a depressant effect has not been confirmed with an in vitro canine papillary preparation until the present study.

The following conclusions were made from the results of these individual studies to evaluate the depressant effects of an endogenous myocardial depressant factor. Whole fresh shock plasma obtained from dogs subjected to hemorrhagic shock, but not control plasma, significantly depressed the rate of muscle shortening and relaxation of isotonically contracting canine papillary muscles but not the ability to shorten against a maximum load. A subsequent study was conducted to generate a depressant substance by incubating canine pancreatic homogenates under shock-like conditions. The homogenates were subsequently fractionated by gel chromatography and various molecular weight fractions evaluated for myocardial depressant effects with an isometric preparation of canine papillary muscle. Significant depressant activity was localized in a chromatographic peak with a molecular weight between 500-1000. The effects of that peak assayed at 37 C and a frequency of 60/minute were to depress developed tension, both the rate of tension developed and the rate of relaxation (decline in tension), and to prolong the time to onset of contraction (latency) and the time to the maximum rate of relaxation. Temperature effects on isometric muscle function were evaluated in a third study. Cooling of

the preparation from 37 C to 27 C caused significant increases in developed or active tension but not resting tension. Each of the respective measurements of the time intervals recorded (LAT, TMVC, TPC, TMVR, and DUR) were all prolonged by cooling. The rate of relaxation was reduced by cooling, but insufficent data were obtained to confirm statistical significance.

The influence of combinations of temperature and frequency on the isotonic response of cardiac muscle was evaluated in the final phase of this project. Cooling had a significant inotropic effect at both a frequency of 12 and 60/minute. An inotropic effect due to a faster frequency of stimulation was only observed at 37 C. Both contraction and relaxation velocity were also increased but only at 27 C. Increasing the frequency antagonized the slowing effect of cooling on all of the time intervals. The control homogenate chromatographic peak had no significant negative effects on muscle function at any combination of temperature or frequency. In contrast, the shock homogenate peak depressed function. This depression was influenced by temperature and frequency. Cooling reduced the magnitude of depression of maximum load.

Relaxation velocity was the single measured parameter which was consistently depressed throughout all phases of this study. Unfortunately, little research has been previously done to better our understanding of the process. Most of the significant studies have only been done in the last decade by Strauer (1973), Strobeck et al. (1975), Tamiya et al. (1977), Brutsaert et al. (1978), and LeCarpentier et al. (1979). The observed load dependence confirmed these prior studies.

Further, the rather unique load-relaxation velocity relationship described by Strauer (1973) and Strobeck et al. (1975) with maximum relaxation at approximately 0.3Po was also observed. If cardiac relaxation in the intact individual is inhibited, then diastolic filling would be reduced. This may be the pathway by which the endogenous depressant factors compromises cardiac output and ultimately leads to irreversible shock. Relaxation would appear to be a parameter for future evaluation of shock factors.

Further studies are necessary before the mechanism of action of the depressant factor can be elucidated. Although it is speculative, these results may suggest that the site of action is intracellular. Such a site is supported by the following observations. A site of action at the sarcolemma could alter the onset of contraction by modifying the action potential or inhibiting the transsarcolemmal pulse of trigger calcium necessary to induce calcium release by the sarcoplasmic reticulum. In the last study in which temperature and frequency effects were evaluated, latency tended to be slower but was significantly altered only at a narrow range of afterloads and then only at low temperature and frequency. However, latency was prolonged in the isometric screen of the chromatographed peaks. Second, relaxation velocity is more depressed than shortening velocity. Either slower cross-bridge turnover or impaired calcium uptake by the sarcoplasmic reticulum could produce such a response. Alternately, if less calcium were released by the sarcoplasmic reticulum, fewer cross-bridges would be formed and less tension would be generated. An uninhibited sarcoplasmic reticulum would sequester the reduced quantity of

sarcoplasmic calcium faster and the rate of muscle lengthening or relaxation would be faster. Contraction velocity is also depressed which suggests that the depressant must affect a common step in both processes. No effect on peak developed isometric tension was observed in the isometric experiments nor was any change in maximum load observed in response to shock plasma. The depressant effect would appear to be independent of the number of cross-bridges formed but alter the rate of formation and turnover.

Higher frequency was shown to antagonize the depression of relaxation velocity caused by the shock peak. This implies that relaxation, which is dependent on the reduction of sarcoplasmic calcium levels, is faster when the calcium is increased by a more rapid frequency. It is possible that the depressant factor could theoretically alter calcium affinity at a key binding site. Mobilization of more calcium at a faster frequency could overcome the affinity change.

Future studies are necessary to further examine these speculative ideas concerning the mechanism of action of the depressant factor. Four distinct approaches need to be followed. First, a metabolic effect of the depressant might be qualitatively assessed by measuring oxygen consumption in response to the depressant. This approached has been previously considered by Dr. F.B. Hembrough. A biochemical approach might include assessing changes in calcium affinity of subcellular organelles or measuring changes in the transient sarcoplasmic calcium levels during a contractile cycle caused by the depressant. Biochemical purification of the depressant factors is perhaps the most essential

future requirement. Although Lefer and Rovetto (1970) have reported that MDF prolonged the duration of the action potential of feline myocardium, only limited electrophysiological studies were conducted. The electrophysiological effects of the depressant need to be reassessed. Finally, modification of the depressant response by various pharmacological agents, especially those which affect intracellular calcium could be evaluated with the current type of in vitro preparation of cardiac muscle.

Use of preparations of isolated cardiac muscle does have definite limitations. Such experiments are extremely time consuming and the number of experiments which can be conducted over a period of time is therefore limited. Great variability between muscles is typically observed not only for canine papillary muscle in the present study, but also for the traditional feline preparation. Roy Goldfarb, who has been cited in this dissertation for publications pertaining to myocardial depressant factor, has personally communicated that the traditional isometric papillary muscle is being abandoned in his future work. He now believes that cell cultures of cardiac muscle will provide a uniform and large population of experimental units. All of the measurements in the present study had to be calculated by hand. This introduces inherent error and illustrates the need to develop an on-line analysis system which incorporates a computer. At least 8-15 hours of hand calculations were necessary for each hour of data collection. However, future studies by the current technology will continue to provide valuable new insights into the role and mechanism of endogenous depressant factors.

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ACKNOWLEDGMENT

I offer my sincere appreciation to Drs. Donald Beitz, Malcolm Crump, Donald Dyer, Mary Helen Greer, and Melvin Swenson for serving on my committee and for the technical and personal support they have offered during this project.

Dr. Frederick Hembrough deserves a very special thanks for serving as my advisor. His patience, kindness, courtesy, and encouragement were invaluable at those times when this research was stalled. It has been a pleasure to have had the opportunity to work with and learn from an individual of his character and quality.

I wish to thank Barb Royer, my technician, for her technical assistance and friendship. The contributions of the other technical personnel and faculty within the Department of Veterinary Physiology and Pharmacology were essential and most appreciated.

A very special thanks to Dr. John and Carol Terry, and to Mary and Arthur Max for their support and assistance. I especially acknowledge the contribution of my mother and father, Dorothy and Elmer Terry. They have been the foundation of my education. No words can adequately express my gratitude for their teaching and their love. My wife Linda and Brian, Emily, and Nathan, my children, deserve recognition for their role. They have sacrificed of themselves and have always forgiven me for the times I was not there. They have been my motivation and have been there when I needed them.

And finally, I acknowledge Karl for the promise that I may begin to keep.